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No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

STUDIES ON ENZYME ACTION.

XXXVIII. The Ester-Hydrolyzing Actions of the Whole Eel.

By HELEN MILLER NOYES, I. LORBERBLATT, and K. GEORGE FALK.

(From the Carnegie Institution of Washington, Station for Experimental Evolution, Cold Spring Harbor, and the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

(Accepted for publication, June 11, 1926.)

The systematic study of the ester-hydrolyzing enzymes of various animal materials has been described in previous communications.[1] Results obtained with the eel are presented in this paper.

Experimental Methods.

Adult eels whose weights ranged from 86 gm. to 337 gm. were used. Immediately after being killed, they were passed twice through a meat chopper. If the solid material was to be tested directly, 0.67 gm. portions were weighed into flasks and 15 cc. water and 1 cc. toluene added to each. If extracts were to be tested, the requisite amounts of water or solution were added to the weighed solid, allowed to extract overnight at room temperature, filtered through paper, suitably diluted, and 15 cc. portions used for the enzyme tests. In every enzyme test the concentration of the eel, in the form of original solid, or of the eel extract corresponded to 44.4 mg. of original material per cc. of mixture or of solution tested. Because of the increases in volume in the dialysis experiments it was necessary to plan for suitable adjustment of the concentrations of the original extracts. Toluene was present throughout in every experiment.

Dialysis was carried out in collodion bags at 14–17° for 15 to 24 hours, either against tap water where the flow was comparatively rapid and the outside liquid not recovered, or against definite volumes

[1] *Cf.* especially *J. Biol. Chem.*, 1924, lix, 183, 213; 1924–25, lxii, 687, 697; *J. Gen. Physiol.*, 1925–26, viii, 75; 1925–26, ix, 651; *J. Am. Chem. Soc.*, 1924, xlvi, 1885; *J. Cancer Research*, 1925, ix, 105; 1926, x, 146.

of distilled water with constant stirring and where the outside liquid was used for enzyme tests.

The enzyme tests were carried out as described in previous communications: ten different esters were used, as shown in the figures and in the table, 3.4 milli-equivalents of each ester, incubation for 22 hours at 37–38°, titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator, and blanks and duplicates in every case.

Results.

The relative hydrolyzing actions of the solid and different extracts on the ten different esters are shown in Figs. 1 and 2. The averages of three closely agreeing series of experiments are given in each curve, the actions on phenyl acetate being largest and placed equal to 100 in each case.

The curves of the relative actions of the eel solids and extracts showed no striking differences either when tested directly or after dialysis against tap water. Minor differences, however, were indicated in the two figures.

The absolute enzyme actions (actual titration values corrected for blanks), are not given in detail for these experiments. Only the general behavior will be stated: The solid material gave the largest actions, the undialyzed aqueous and 50 per cent glycerol extracts gave much the same actions but somewhat less than the solid, while the undialyzed 10 per cent sodium chloride extracts gave the smallest actions. On dialysis, the absolute actions of the various extracts decreased except for the 10 per cent sodium chloride extracts where the actions on phenyl acetate, glyceryl triacetate, and methyl butyrate were larger for the dialyzed than for the undialyzed extracts.

Table I shows the results of a complete experiment (Experiment F20) in which the enzyme tests were carried out with the solid eel material and the various extracts directly, on these same mixtures after dialysis against distilled water, on the dialysates, and on the dialyzed mixtures to which the dialysates were added. The concentration of each mixture tested was equivalent to 44.4 mg. of original material per cc. of solution tested. The actual titration values, cor-

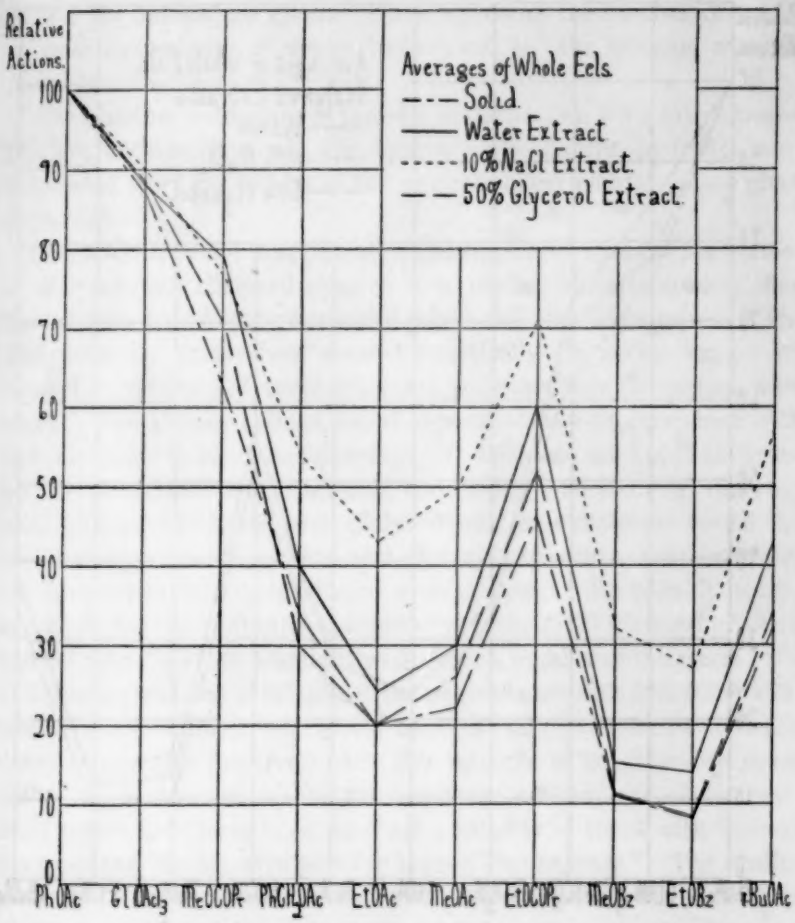

FIG. 1. Averages of relative ester-hydrolyzing actions of solid eel preparation and of undialyzed extracts.

The 10 per cent NaCl extracts were diluted for the tests to contain 3.3 per cent NaCl; the 50 per cent glycerol extracts to contain 16.7 per cent glycerol. The curves of the relative actions of the solids and of the glycerol extracts were essentially the same; compared to these curves the aqueous extracts showed somewhat greater actions on the esters following phenyl acetate and glyceryl triacetate, and the 10 per cent NaCl extracts considerably greater for the last seven esters in comparison with the first three.

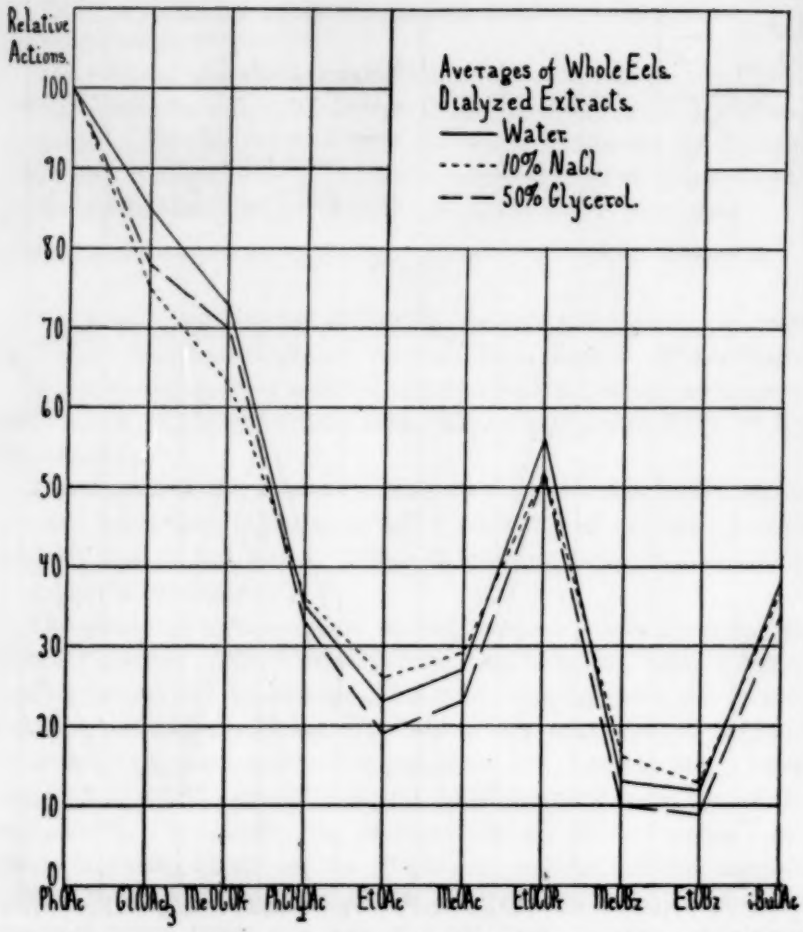

FIG. 2. Averages of relative ester-hydrolyzing actions of eel extracts dialyzed against tap water.

The curves of the three dialyzed extracts were essentially the same. Compared with the undialyzed extracts, the curves for the dialyzed aqueous and 50 per cent glycerol extracts showed no change, while for the 10 per cent NaCl extracts decreased actions relative to that on phenyl acetate were found with the remaining esters for the dialyzed extracts.

rected for blanks, are given. These represent the number of tenths of milli-equivalents of esters hydrolyzed by the enzyme material under the conditions of the tests.

The absolute values are of interest in connection with the curves of relative actions shown in the figures. The figures in italics were calculated from the results of the separate experimental values given in the table.

One striking result is apparent. The calculated sums of the actions of dialysate and dialyzed solution in a number of cases are less than the actions found experimentally when the two were mixed. The dialysates by themselves showed practically no action except on phenyl acetate and glyceryl triacetate and even here the actions were small. The greater actions found experimentally as compared with the calculated include the following: (f) Dialyzed solid plus dialysate for the butyrates, benzyl acetate, and isobutyl acetate; (g) dialyzed solid plus liquid filtered from dialyzed solid plus dialysate for all the esters except phenyl acetate and glyceryl triacetate, but especially the butyrates; (h) liquid filtered from dialyzed solid plus dialysate, especially for the butyrates and isobutyl acetate; (t) dialyzed glycerol extract plus dialysate, small differences for a number of the esters.

These separations of certain of the enzyme materials into two parts, one of which is much less active than the original mixture and the other practically inactive, while the mixture is considerably more active than the sum of the two separate actions, are of interest. Such separations have been reported a number of times with various enzymes and the inactive portion termed "co-enzyme." The results presented here show that the solvent used influences the possibility of the separation and that the action of the co-enzyme may be very different for different substrates. No explanation is at hand for the action of the co-enzyme, but its greater influence on the hydrolysis of the butyric esters may possibly be of significance.

The absolute actions given in Table I supplement the relative actions shown in Figs. 1 and 2.

The ester-hydrolyzing actions of the eel preparations were studied also at 14–17°. It was found that the actions on some of the esters in 22 hours were greater at the lower temperature than at 37–38°. This was shown to be due, in the main, to the combination, or opposing

TABLE I.

Ester-Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced from the Indicated Esters by Eel Preparations.

	PhOAc	Gl(OAc)$_3$	MeOCOPr	PhCH$_2$OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	i-BuOAc
(a) Original solid.	4.82	4.00	2.86	1.81	0.86	1.02	2.13	0.42	0.41	1.53
(b) Solid-dial. *vs.* dist. water.	2.42	1.90	1.04	0.40	0.25	0.21	0.59	0.13	0.15	0.36
(c) Liquid in bag filtered from solid after dial. *vs.* dist. water.	2.05	1.69	1.49	0.77	0.41	0.50	1.07	0.21	0.19	0.75
(d) Dialysate from solid.	0.53	0.27	0.11	0.04	0.05	0.10	0.04	0.02	0.00	0.02
(e) Solid (b) + liquid (c).	3.88	3.15	2.47	0.99	0.66	0.75	1.60	0.31	0.33	0.97
(e) Solid (b) + liquid (c) (calc.).	*4.47*	*3.59*	*2.53*	*1.17*	*0.66*	*0.71*	*1.66*	*0.34*	*0.34*	*1.31*
(f) Solid (b) + dialysate (d).	2.85	2.26	1.49	0.60	0.32	0.42	1.11	0.27	0.25	0.60
(f) Solid (b) + dialysate (d) (calc.).	*2.95*	*2.17*	*1.15*	*0.44*	*0.30*	*0.31*	*0.63*	*0.15*	*0.15*	*0.38*
(g) Solid (b) + liquid (c) + dialysate (d).	4.29	3.48	3.28	1.55	1.02	1.07	2.26	0.66	0.50	1.49
(g) Solid (b) + liquid (c) + dialysate (d) (calc.).	*5.00*	*3.86*	*2.64*	*1.21*	*0.71*	*0.81*	*1.70*	*0.36*	*0.34*	*1.13*
(h) Liquid (c) + dialysate (d).	2.90	2.41	2.21	1.14	0.66	0.84	1.82	0.46	0.38	1.33
(h) Liquid (c) + dialysate (d) (calc.).	*2.58*	*1.96*	*1.60*	*0.81*	*0.46*	*0.60*	*1.11*	*0.23*	*0.19*	*0.77*
(i) Water extract.	3.66	3.07	2.99	1.53	0.98	1.13	2.35	0.60	0.54	1.57
(j) Water extract dial. *vs.* dist. water.	2.81	2.42	2.06	1.11	0.74	0.84	1.62	0.47	0.42	1.17
(k) Dialysate from water extract.	0.20	0.13	0.02	0.03	0.05	0.08	0.03	0.00	0.02	0.00
(l) Extract (i) + dialysate (k).	3.74	3.20	3.03	1.55	1.00	1.17	2.36	0.73	0.51	1.55
(l) Extract (i) + dialysate (k) (calc.).	*3.86*	*3.20*	*3.01*	*1.56*	*1.03*	*1.21*	*2.38*	*0.60*	*0.56*	*1.57*
(m) Dial. extract (j) + dialysate (k).	3.27	2.75	2.62	1.41	0.85	0.99	2.09	0.66	0.50	1.46
(m) Dial. extract (j) + dialysate (k) (calc.).	*3.01*	*2.55*	*2.08*	*1.14*	*0.79*	*0.92*	*1.65*	*0.47*	*0.44*	*1.17*
(n) 10 per cent NaCl extract.	1.95	1.74	1.49	1.02	0.89	0.96	1.36	0.71	0.56	1.05
(o) 10 per cent NaCl extract dial. *vs.* running water.	2.38	1.87	1.56	0.89	0.66	0.74	1.33	0.45	0.32	0.88

(p) 50 per cent glycerol extract.	3.96	3.25	2.62	1.16	0.76	0.92	1.92	0.41	0.31	1.14
(q) Glycerol extract dial. *vs.* dist. water.	3.04	2.54	2.13	1.05	0.59	0.73	1.63	0.34	0.28	1.08
(r) Dialysate from glycerol extract.	0.57	0.25	0.11	0.03	0.01	0.04	0.06	0.04	0.01	0.00
(s) Extract (p) + dialysate (r).	4.17	3.74	2.65	1.21	1.03	1.09	2.01	0.49	0.31	1.29
(*s*) *Extract* (*p*) + *dialysate* (*r*) (*calc.*).	*4.53*	*3.50*	*2.73*	*1.19*	*0.77*	*0.96*	*1.98*	*0.45*	*0.32*	*1.14*
(t) Dial. extract (q) + dialysate (r).	3.96	3.13	2.41	1.06	0.71	0.88	1.83	0.39	0.35	1.16
(*t*) *Dial. extract* (*q*) + *dialysate* (*r*) (*calc.*).	*3.61*	*2.79*	*2.24*	*1.08*	*0.60*	*0.77*	*1.69*	*0.38*	*0.29*	*1.08*

natures, of two actions; one, the different rates of inactivation of the enzyme at the two temperatures, and the other, the different hydrolytic actions of the enzyme at the same two temperatures. These results, as well as others bearing on the problem of enzyme actions at different temperatures have been presented elsewhere,[2] and will therefore only be referred to in this connection.

SUMMARY.

The hydrolyzing actions of various preparations of the adult eel were studied on ten esters in the usual way. The results are presented in the form of curves for the relative actions and in a table for the absolute actions obtained in one complete experiment.

The separation of the enzyme material in some cases into an active portion and a co-enzyme, the mixture showing greater actions on some esters than the sums of the individual actions, is described and discussed.

[2] Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Biol. Chem.*, 1926, lxviii, 135.

MICRURGICAL STUDIES IN CELL PHYSIOLOGY.

II. The Action of the Chlorides of Lead, Mercury, Copper, Iron, and Aluminum on the Protoplasm of Amœba proteus.

By PAUL REZNIKOFF.

(From Cornell University Medical College, New York.)

(Accepted for publication, June 22, 1926.)

The effects on protoplasm of some of the cations found in physiological systems have been reported in a previous communication (1). This study utilized the micrurgical technique and the advantages of such a method over that of simple immersion were pointed out in detail. Only by combining the results of injecting substances into the living cell with those obtained by the immersion method can one obtain proper conceptions of such important physiological problems as those of permeability, site of toxic action, antagonism of ions, and protoplasmic consistency.

Our knowledge of the action of heavy metals has been limited because of the difficulty in localizing the effect of salts in definite parts of the cell. The rapidity with which a cell or tissue reacts has usually been considered to mean ease of penetration (2, 3). But this is not necessarily the case. Even where some change has been noted inside the cell one cannot be certain that it might not be due to a surface effect which involved a secondary change within the interior or that it was not caused by the abstraction of some substance from the interior of the cell.

In a review of the work dealing with the action of disinfectants, especially $HgCl_2$, Cohen (4) points out that previous contributions deal mainly with the course of the process and not with the mechanism concerned. The micrurgical method is especially adapted to a study of the mechanism of the reactions between salts and protoplasm because of the ease with which substances can be brought into direct contact with definite parts of the cells. The importance of many

of the metals in certain physiological (5–7) and pathological (8–10) problems suggested their study by means of micrurgy. With the aid of microdissection and injection, therefore, the effects of the chlorides of lead, mercury, copper, iron, and aluminum on *Amœba proteus* were investigated. It is the purpose of this paper to present the results of immersing and tearing amebæ in solutions of these salts and of injecting such solutions into these cells.

The manipulation of the apparatus, the general experimental technique, and the terms used, have been fully described in our former paper (1) and the reader is referred to that report for the details of the procedure. The only general modification of the method used in the experiments described in this paper was that in most of them fewer amebæ were used. This seemed justifiable because of the relatively constant quantitative results with several thousand amebæ from the same stock in former experiments.

I.

Immersion Experiments.

$PbCl_2$.—$PbCl_2$ undergoes gradual hydrolysis in solution, with increase in acidity, and not until a dilution of M/5500 is reached can a solution be maintained at pH 5. If amebæ are immersed in decreasing concentrations of such solutions of $PbCl_2$, they undergo a slow change in shape. They gradually retract their pseudopodia and assume the elliptical form of the so called *Limax* type. The surface is stiffened and the ameba becomes very sluggish. Finally the cell becomes rounded and then dies. The curve labelled $PbCl_2$ in Fig. 1 illustrates the relatively slow action of $PbCl_2$. It is toxic eventually in even very great dilutions. Amebæ cannot survive longer than 5 days until a dilution of M/22,000,000 is reached.

$HgCl_2$.—Amebæ immersed in $HgCl_2$ die very rapidly compared to those in most of the other salts (Fig. 1). In solutions stronger than M/8000 they are converted into small round masses. In solutions ranging from M/8000 to M/250,000 the plasmalemma breaks and the contents begin to scatter but solidify rapidly. Fig. 1 shows that the curve of toxicity for $HgCl_2$ is relatively steep. A solution of M/125,000 is toxic in 1 hour, but amebæ survive more than 5 days in M/500,000. That the immediate effect may not be due entirely to

the acidity which develops when $HgCl_2$ is dissolved in water is shown by the fact that amebæ can live normally in water at pH 6 but die rapidly in the presence of an M/64,000 solution of $HgCl_2$ of the same pH.

$CuCl_2$.—The reaction of an M/1000 solution of $CuCl_2$ is pH 5.5 1 day after the salt is dissolved. After 3 days the acidity increases to pH 4.8. Immersion of amebæ in these solutions of $CuCl_2$ causes the amebæ to become rounded and the contractile vacuole to increase in size. During the first 3 days of immersion amebæ die in a considerable range of dilutions (Fig. 1). In solutions weaker than M/4,100,000 toxicity decreases abruptly.

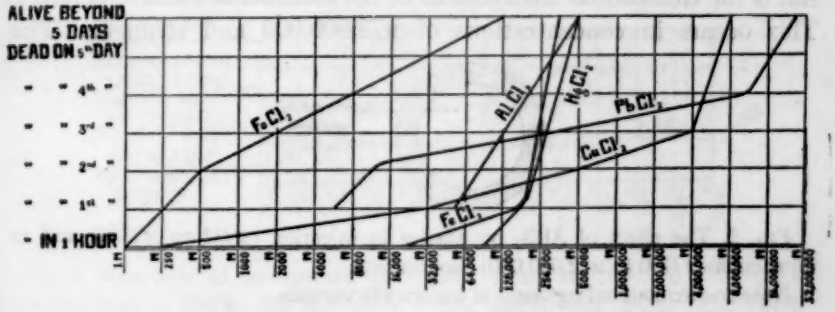

FIG. 1. Viability of amebæ immersed in decreasing concentrations of salt solutions ($PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $AlCl_3$).

$FeCl_2$.—The acidity of solutions of $FeCl_2$ increases rapidly on standing, the greatest amount of change, however, occurring early. For example, a solution as dilute as M/66,000 changes from pH 6.2 to pH 5 in 4 days. These solutions of $FeCl_2$ cause immersed amebæ to become rounded and the crystalloid granules within the ameba become blackened. $FeCl_2$ is relatively non-toxic during the first 2 days of immersion after which time the toxicity increases rapidly (Fig. 1). Thus, amebæ can live in a more dilute solution than M/500 for 2 days but cannot live for 5 days until an M/128,000 solution is reached.

$FeCl_3$.[1]—Solutions of $FeCl_3$ gradually increase in acidity (*e. g.* M/325,000 has a reaction of pH 6.2 when first made and is more acid

[1] In preparing solutions of $FeCl_3$ the molecular weight was taken as 540.44 (Fe_2Cl_6, $12H_2O$).

than pH 5 in 5 days). $FeCl_3$ is much more toxic but produces the same visible effect as the salt of divalent iron. The amebæ become rounded and the crystalloid granules blacken. The maximum toxicity is approached after 1 day of immersion (Fig. 1). Subsequently the curve of toxicity rises more steeply than for any of the other salts used in these experiments.

$AlCl_3$.—Solutions of $AlCl_3$ are acid in reaction over a considerable range (an M/64,000 solution has a pH of 5). This salt, in concentrations of M/400 and higher, causes the amebæ to become round rapidly. In solutions from M/400 to M/32,000 the amebæ continue to move about until their surfaces break. The most striking effect of the salt is the tremendous enlargement of the contractile vacuole (Fig. 2). This occurs in concentrations of M/2,000,000 and stronger. The

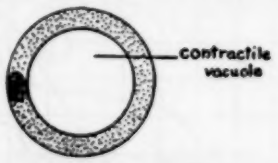

FIG. 2. The effect of $AlCl_3$ on amebæ in injection (M/32 to M/250) and in immersion (M/400 to M/2,000,000) experiments.

Note tremendous enlargement of contractile vacuole.

granules are pushed against the plasmalemma by the distended vacuole. In very dilute solutions, in which the amebæ may live beyond 5 days, the large contractile vacuole may persist for 2 or 3 days but then decreases to its normal size and the ameba recovers completely. The curve of toxicity of $AlCl_3$ is fairly steep (Fig. 1).

II.

Injection Experiments.

$PbCl_2$.—In solutions stronger than M/1000, $PbCl_2$ is changed rapidly into the insoluble carbonate by the abstraction of CO_2 from the air. The injection of $PbCl_2$ in concentrations ranging from M/1000 to M/20,000 (Fig. 3) causes the gradual appearance of an irregular, glassy mass containing very few granules. The streaming movements in the rest of the ameba are very active and the glassy mass is

extruded. A second injection immediately after the first results in no solidification. If a large amount is introduced, the surface of the cell may break. If a second injection is made 20 minutes or more after the first, a second solidification and extrusion of the affected region may occur. Subsequent injections have no effect on the viability of the ameba, whether extrusions do or do not occur.

The effect of $PbCl_2$ in causing a delayed coagulation after the first injection and no coagulation after an immediate second injection, suggest that in the reaction between $PbCl_2$ and the protoplasm the lead uses up some cellular constituent which gradually forms anew in the cell. This harmonizes with the suggestion made by Aub and Rez-

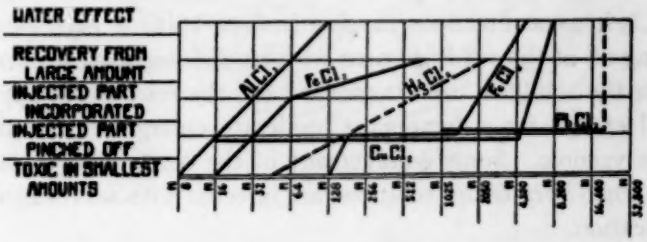

FIG. 3. Recovery of protoplasm of amebæ from injection of decreasing concentrations of $PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, and $AlCl_3$. The curve labelled $HgCl_2$ is represented by a broken line because no pinching off of the region injected occurs. The broken line in the curve labelled $PbCl_2$ indicates an abrupt transition from the pinching off of the coagulated region to the water effect. No intermediate stages are seen.

nikoff (11) that lead, in the small concentrations used in these experiments, unites with the phosphates or carbonates of the cell. The delay in the formation and the peculiar glassy appearance of the solidified mass in the cytoplasm also point to a different type of reaction between protoplasm and lead than those obtained with the other coagulating ions studied.

$HgCl_2$.—A solution of M/5 to M/50 $HgCl_2$ causes an immediate solidification of the internal protoplasm (Fig. 3). With solutions of M/100 to M/1600 the surface breaks and some of the contents of the ameba flows out. Small amounts of M/300 $HgCl_2$ cause a disruption of the surface with subsequent recovery. Injections of small amounts of M/600 produce no break and the ameba readily recovers from the

breaks caused by larger injections. A moderate quantity of M/1600 causes no break but the ameba may pinch off the somewhat solidified injected region. An injection of an M/2400 solution even in large quantities causes no disruption of the plasmalemma.

The results indicate that both very high and very low concentrations of $HgCl_2$ act principally on the interior of the cell. Moderate concentrations erode the plasmalemma.

$CuCl_2$.—The injection of solutions of $CuCl_2$ causes a solidification of the affected region and a disintegration of the adjacent surface. With M/16 the ameba can pinch off the affected region (Fig. 3). Stronger solutions solidify the entire cell. Not until M/8000 is reached does this solidification process cease. Each successive injection of $CuCl_2$ in concentrations ranging from M/16 to M/8000 causes the pinching off of the solidified area with its disintegrated surface.

After the solidified area is pinched off, the remnant is apparently normal except for a temporary moderate enlargement of the contractile vacuole. Some enlargement of the contractile vacuole also occurs when very dilute solutions are injected with no resulting local solidification.

$FeCl_2$.—Solutions of $FeCl_2$ stronger than M/32 solidify the internal protoplasm with which they come into direct contact (Fig. 3). With M/32 the affected portion is usually pinched off. M/64 causes a quiescence and partial solidification of the injected region, which is subsequently reincorporated by the active portion of the ameba. Large amounts of M/512 may sometimes cause death, but moderate quantities of M/32 to M/64 as a rule are not fatal. The method by which the ameba constricts off the solidified part when $FeCl_2$ is injected is somewhat different from that observed with most other solidifying agents. Instead of a sharp constriction between the injured and healthy portions (1), the ameba forms a line of demarcation between the healthy and affected area and the living part flows around the solidified region so that the latter lies in a deep depression and is slowly extruded as the depression is everted (Fig. 4). The surface of the extruded region has no definite pellicle in some places. The living portion appears normal except for a slightly enlarged contractile vacuole.

$FeCl_3$.—$FeCl_3$ is much more toxic than the divalent chloride of iron to the internal protoplasm (Fig. 3). A small injection of M/160 solidifies almost the entire ameba and the streaming movements of the unaffected region, which are apparently attempts to pinch itself off, soon cease and the whole cell is killed. From M/320 to M/1280 pinching off usually occurs after each injection. Occasionally, after the introduction of M/320 the ameba may incorporate the affected mass. With M/5120 an extrusion of a small mass from the injected region may occasionally occur after repeated injections. $FeCl_3$ causes the ameba to become sluggish and no typical water effect (1) is obtained until a dilution of M/10,240 is reached. Im-

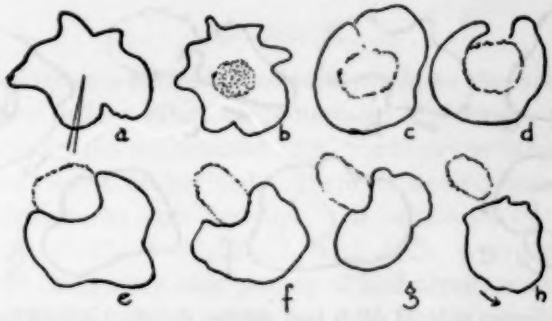

FIG. 4. Injection of M/32 $FeCl_2$ into ameba; *a*, before injection; *b*, solidification of the region injected; *c*, *d*, *e*, *f*, and *g*, extrusion by sliding around solidified area; *h*, complete separation of living remnant from dead area.

mediately after injection the vacuole of the ameba enlarges to some extent.

$AlCl_3$.—The introduction of $AlCl_3$ has a very striking effect on the ameba. In strengths of M/16 and stronger the injected area is solidified (Fig. 3). M/8 affects the entire ameba but when a solution of M/16 is introduced only the portion injected is solidified and is subsequently pinched off by the unaffected part of the ameba. The injection of M/32 also causes solidification, and pinching off may occur if the injection is made near the edge of the cell. However, in many such cases the ameba may reincorporate the affected mass after it is almost separated from the living portion, as though it were a foreign body. The solidified portion, held by a narrow band is finally en-

gulfed by the ameba and is soon completely absorbed. The various steps of this process are illustrated in Fig. 5. One of the most marked features of the injection of $AlCl_3$ in concentrations ranging from M/32 through M/250 is the tremendous enlargement of the contractile vacuole (Fig. 2). This is identical with the results obtained in the immersion experiments. The ameba recovers from this condition, which may last from a few hours to several days, depending upon the concentration of $AlCl_3$ injected. The similarity in appearance of the internal protoplasm in immersion and injection experiments indicates a high degree of permeability which is in accord with the findings of Michaelis (12).

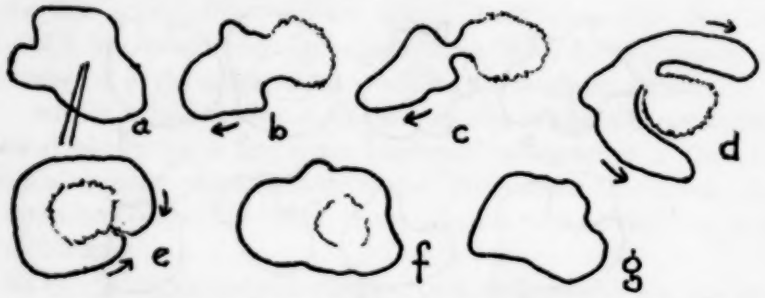

FIG. 5. Injection of M/32 $AlCl_3$ into ameba; *a*, before injection; *b* and *c*, uninjured portion flowing away from injected area and beginning to pinch it off; *d*, almost complete pinching off and beginning of engulfment of injured region; *e*, completion of engulfment; *f*, incorporation; *g*, return to normal state.

III.

Tearing Experiments.

Because of the marked toxicity of even dilute solutions of the heavy metal salts to immersed amebæ, it is very difficult to maintain them alive and in good condition long enough to react visibly to a tearing operation. Thus, in $HgCl_2$ and $FeCl_3$ the rapidity of action of the salts is so great compared to that in salts like $AlCl_3$ and $PbCl_2$ that the amebæ are dead before the needles can be brought into use. Therefore the results of these experiments merely show the dilution at which the immersed amebæ remain alive long enough to react to a marked trauma of the needle. This, of course, varies with the rate of the action of the salt on the plasmalemma.

With this limitation in view the following table indicates the reparability of the torn surfaces of amebæ immersed in these salts.

Salt.	Greatest concentration of salts in which repair of torn plasmalemma takes place.
$AlCl_3$	M/320
$PbCl_2$	M/1,000
$FeCl_3$	M/2,000
$CuCl_2$	M/3,200
$FeCl_2$	M/10,200
$HgCl_2$	M/24,000

DISCUSSION.

These experiments present some evidence as to the actual mechanism involved in the effect on protoplasm of the salts tested. An attempt to group the entire action of all the heavy metals into a common process cannot be justified. There is, however, one common feature which merits consideration. All salts used in this work, $PbCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, and $AlCl_3$, hydrolyze to form strong acids. Moreover, this process of hydrolysis takes place over an extended period of time. This has been studied for lead salts by von Ende (13). Coincident with the increase in acidity the amebæ gradually die and it is probable that this liberation of acid is at least one important factor in their death.

The marked toxicity of these salts when amebæ are immersed in them as compared with the results obtained when the salts are injected into the amebæ indicates that the lethal action of these substances is on the surface of the cell. In no case was the solution sufficiently acid to produce an effect on the internal protoplasm by its acidity alone. This has been shown by experiments previously reported (1), in which repeated injections of a solution of HCl at a reaction of pH 3 had no effect on the internal protoplasm but immersion in HCl at a reaction of pH 5 was lethal in a short time. This lends further support to the view that the action of the salt when injected is due to the cation alone, and that when the ameba is immersed in the solution the effect on the surface is due to the presence of the acid, which is being constantly produced, as

well as to the metal cation. It might, however, be suggested that the buffers in a cell can effectively neutralize any acid formed by hydrolysis. But the possibility must be considered that local effects may occur, for example on the surface, and cause irreversible changes before buffering of the acid takes place. This has been suggested by Aub and Reznikoff (11) as a possible mechanism in the action of lead on cells.

That the relative non-toxicity of the salts on the internal protoplasm may be due to their outward diffusion is not probable. No direct evidence for such outward diffusion was found in the case of NaCl (1), and the salts used in these experiments form much more stable compounds with protoplasm than does NaCl.

Some of these salts seem to have an effect on a specific part of the cell, depending upon their concentration. This is particularly true for $HgCl_2$ as has been found also by Bechhold (14) with red blood cells and by MacInnes (15) with *Aspergillus niger*. There is an indication, therefore, that various chemical combinations may be formed between the toxic substance and the different constituents of the cell depending upon the relative concentration of the toxic agent used. Krahé (16) suggests that the action of $HgCl_2$ is due not only to its ionization but, in certain concentrations, to its lipoid solubility.

The gradual increase in toxicity of $FeCl_2$ in the immersion experiments may be associated with its gradual oxidation to the trivalent iron salt which is rapidly toxic. In this connection it is interesting to note that Buschke, Jacobsohn, and Klopstock (17) believe that the "oligodynamic" action of metals depends to a great extent on the ionization of their salts and on an oxidation process.

$PbCl_2$, in the concentrations used in these experiments, probably acts by uniting with the phosphates or carbonates of the cell and thus liberating free acid. Such a secondary reaction is indicated by the slow rate of toxicity.

A striking feature brought out by these experiments is the greater variation in the viability of different amebæ in these solutions when compared to that which occurs in salts such as NaCl, KCl, $CaCl_2$, and $MgCl_2$ (1).

In attempting to determine the mode of action of a toxic substance on a cell it is necessary to consider all the possible mechanisms in-

volved. A toxic agent may (*a*) affect the plasma membrane only, (*b*) affect both the plasma membrane and the internal protoplasm, (*c*) leave the plasma membrane unharmed and injure the internal protoplasm, or (*d*) may not enter the cell but affect it by abstracting a necessary constituent. In considering the third possibility, (*c*), there is no evidence available, so far, to support the belief that a substance may pass through the plasmalemma in a non-toxic form and by some chemical alteration may change into a toxic form inside the cell or may be harmful to the internal protoplasm only. A consideration of the other possibilities, (*a*), (*b*), (*d*), suggests that a substance either abstracts a necessary constituent from the cell or primarily affects its surface. All visible evidence obtained so far points to the fact that toxic agents affect the surface of the immersed cell. So consistent is this result that the suggestion may be made that the maintenance of the surface membrane in a normal state is necessary for the life of the cell.

CONCLUSIONS.

I. *Plasmalemma.*

1. The order of toxicity of the salts used in these experiments on the surface membrane of a cell, taking as a criterion viability of amebæ immersed in solutions for 1 day, is $HgCl_2$, $FeCl_3 > AlCl_3 > CuCl_2 > PbCl_2 > FeCl_2$.

Using viability for 5 days as a criterion, the order of toxicity is $PbCl_2 > CuCl_2 > HgCl_2 > AlCl_3 > FeCl_3 > FeCl_2$.

2. The rate of toxicity is in the order $FeCl_3 > HgCl_2 > AlCl_3 > FeCl_2 > CuCl_2 > PbCl_2$.

3. The ability of amebæ to recover from a marked tear of the plasmalemma in the solutions of the salts occurred in the following order: $AlCl_3 > PbCl_2 > FeCl_2 > CuCl_2 > FeCl_3 > HgCl_2$.

II. *Internal Protoplasm.*

4. The relative toxicity of the salts on the internal protoplasm, judged by the recovery of the amebæ from large injections and the range over which these salts can cause coagulation of the internal protoplasm, is in the following order: $PbCl_2 > CuCl_2 > FeCl_3 > HgCl_2 > FeCl_2 > AlCl_3$.

5. $AlCl_3$ in concentrations between M/32 and M/250 causes a marked temporary enlargement of the contractile vacuole. $FeCl_2$, $FeCl_3$, and $CuCl_2$ produce a slight enlargement of the vacuole.

6. $PbCl_2$, in concentrations used in these experiments, appears to form a different type of combination with the internal protoplasm than do the other salts.

III. *Permeability.*

7. Using the similarity in appearance of the internal protoplasm after injection and after immersion to indicate that the surface is permeable to a substance in which the ameba is immersed, it is concluded that $AlCl_3$ can easily penetrate the intact plasmalemma. $CuCl_2$ also seems to have some penetrating power. None of the other salts studied give visible internal evidence of penetrability into the ameba.

IV. *Toxicity.*

8. The toxic action of the chlorides of the heavy metals used in these experiments, and of aluminum, is exerted principally upon the surface of the cell and is due not only to the action of the metal cation but also to acid which is produced by hydrolysis.

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A SIMPLE ELECTRO-ULTRAFILTER.

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It is frequently desirable to free colloidal solutions of electrolytes, but the process of ordinary dialysis is very slow and often cumbersome. Although the rate of diffusion of electrolytes out of the solution may be increased by means of electrophoresis, the limited choice of membranes available for use with the existing apparatus often leads to unequal rates of passage of the ions to the respective electrodes, and to greater or less marked changes in the hydrogen ion concentration of the solution subjected to dialysis.

We have encountered these difficulties in attempting to dialyze the solutions containing bacteriophage and have been successful in overcoming them by combining the principle of ultrafiltration with electrophoresis.

Our apparatus consists essentially of three concentric chambers, of which the middle one is formed by the collodion membranes deposited on the surfaces of two alundum thimbles (8 and 9, Fig. 1, *a*). The other two chambers serve for removing the dialyzed electrolytes from the membrane by means of a stream of cold water.

The different parts of the dialyzing apparatus are as follows:

(1) A glass tube[1] (3 mm. diameter), supplying distilled water from the reservoir (20) to the inner surface of the alundum thimble (8) carrying the positively charged membrane.

(2) A rubber stopper securing the position of the glass tube (1) in place.

(3) A glass T-tube draining by suction into receptacle (21).

(4) A rubber collar securing an air-tight connection between the T-tube (3) and the carbon (5).

(5) A soft core arc lamp carbon 12 mm. in diameter and 150 mm. long, bored

[1] This tube is slightly flared out at the lower end and carries a rubber washer to make an air-tight connection at the lower end of the carbon, so that at this point the glass tube is patent, but the hollow carbon (5) is not.

out to admit freely the glass tube (1) and connected with the positive wire of the electric current. 60 mm. from the slightly tapered lower end of the hollow carbon, a 2 mm. hole permits the return flow of water from the inner chamber around the glass tube (1) and up to the collecting flask (21).

(6) A rubber stopper, No. 13, shelved at its lower end so that a projection 10 mm. deep and approximately 34 mm. in diameter is left to accommodate the outer alundum thimble (9).

(7) A piece of rubber tubing making an air-tight connection between the carbon and the alundum thimble (8).

(8) A Norton alundum thimble, No. 10472 RA 360, 17 mm. in diameter and 80 mm. long. After this part of the apparatus has been assembled, a collodion membrane of the desired degree of permeability is deposited, under pressure, on the outer surface of the thimble (8). For this purpose the T-tube (3) is connected with the vacuum pump, the rubber tube at (1) is clamped off, and the thimble, while under negative pressure, is dipped into the solution of collodion in glacial acetic acid[2] for 60 seconds. The connection with the vacuum pump is now broken, the excess of collodion is allowed to run off, and the coated surface is placed into running warm water to coagulate the collodion and to remove the acid. The removal of the last traces of acid is most quickly accomplished by assembling the entire apparatus and using electrophoresis. The membranes can be used repeatedly, provided they are kept in water when not in use. Their permeability remains practically constant if they are not allowed to dry. To renew the membrane it is necessary to remove the thimble, allow it to dry in air, and when thoroughly dry to incinerate it in the open flame.

(9) Norton alundum thimble, No. 6406 RA 360, 34 mm. in diameter, 100 mm. long. The inner surface of the thimble must be glazed for a distance of 10 mm. from the top, and the collodion membrane that is deposited upon its inner surface ends at this glazed rim. For this purpose, the thimble is fitted by means of a suitably cut rubber stopper into a cylindrical funnel, and attached to the vacuum pump so as to apply negative pressure to the outside of the alundum. The thimble is then filled with collodion for 60 seconds, the collodion is poured off, and warm water used as before. The glazed surface will be found to slip easily over the projecting portion of the rubber stopper (6).

(10) Brass wire gauze, 120 mesh, tightly wrapped around the thimble (9) and held in place by clips connecting it with the lead (12) from the negative pole of the electric circuit.

(11) A glass tube with rubber stopper at each end, carrying the negative lead (12).

(12) Lead from the negative pole of the circuit.

(13) Glass tube carrying distilled water from the reservoir (19) to the middle chamber containing material for dialysis.

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[2] Bechhold, H., and Gutlohn, L., *Z. ang. Chem.*, 1924, xxxvii, 494.

(14) Thermometer.

(15) A glass receptacle serving as an outer chamber and connected with receptacle (21) by a rubber tube forming a movable joint.

(16) Overflow carrying away the ions migrating to the negative electrode.

(17) Stop-cock for emptying glass receptacle (15).

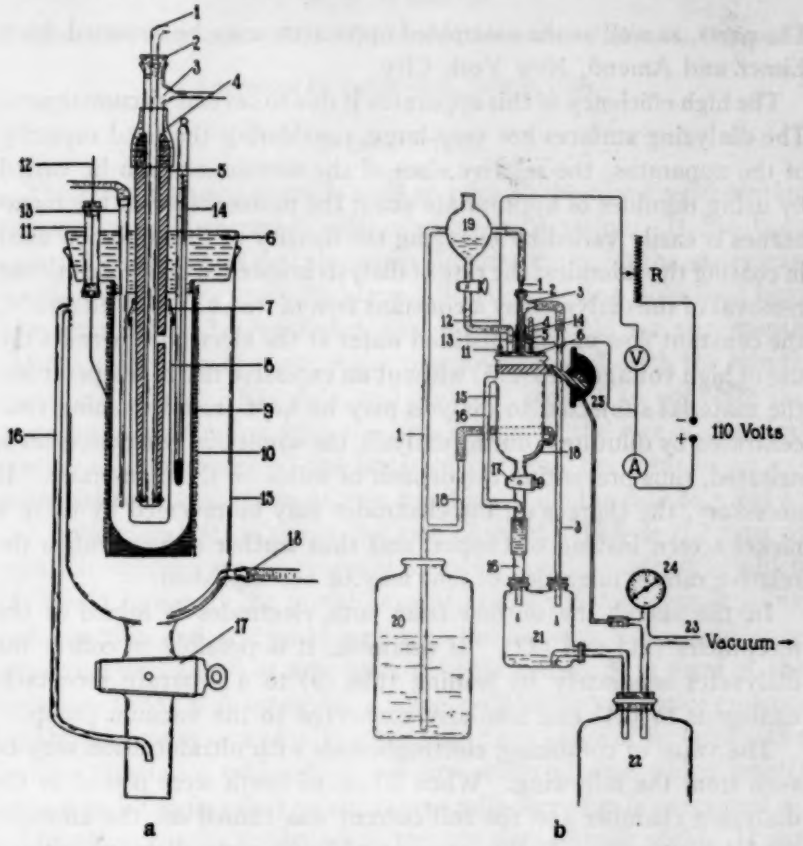

FIG. 1.

(18) Inlet for distilled water.

(19) Fig. 1, *b*. Distilled water reservoir for middle chamber.

(20) Distilled water reservoir for inner and outer chambers.

(21) Intermediary receptacle for dialysates.

(22) Vacuum drainage carboy.

(23) To vacuum pump.

(24) Vacuum gauge.
(25) Vacuum motor (windshield wiper).
(*A*) Weston ammeter (Model 280).
(*V*) Weston voltmeter, with switch (Model 280).
(*R*) Sliding resistance, 650 ohms.

The parts, as well as the assembled apparatus, may be obtained from Eimer and Amend, New York City.

The high efficiency of this apparatus is due to several circumstances. The dialyzing surfaces are very large, considering the total capacity of the apparatus; the relative sizes of the membranes can be varied by using thimbles of appropriate sizes; the permeability of the membranes is easily varied by changing the density of the collodion used in coating the thimbles; the rate of dialysis is speeded up by a constant removal of the dialysate by a constant flow of water at each electrode; the constant flow of cold, distilled water at the electrodes permits the use of high voltage (110–115) without an excessive rise in temperature; the material subjected to dialysis may be kept from becoming concentrated by diluting it during dialysis; the whole dialyzing chamber is agitated, thus preventing the deposit of solids on the membrane. If necessary, the charges on the electrodes may be reversed by using a nickel screen instead of copper, and thus further adjustment in the relative rate of migration of ions may be accomplished.

In the sketch the outflow from both electrodes is mixed in the receptacles (21) and (22). If desirable, it is possible to collect the dialysates separately by leading tube (3) to a separate receptacle analogous to (21) and similarly connected to the vacuum pump.

The value of combining electrophoresis with ultrafiltration may be seen from the following. When 20 cc. of broth were placed in the dialyzing chamber and the full current was turned on, the ammeter read 4.5 amperes. If this amount of broth were dialyzed without current, it would require 3 hours to increase its resistance to the passage of current sufficiently to give an ammeter reading of 0.016. When dialysis was combined with electrophoresis, a reading of 0.013 amperes was reached in 45 minutes.

INTERPRETATION OF THE LACTATION CURVE.

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(Accepted for publication, June 9, 1926.)

INTRODUCTION.

The term lactation curve is used to refer to the curve representing the rate of milk secretion with advance in lactation. It has been known for some years that the group lactation curve for the dairy cow is of a descending exponential type. Sturtevant[1] first published herd data from which he concluded that the milk yield for any month showed a decrease of about 9 per cent as compared with the month preceding. Brody, Ragsdale, and Turner,[2] seeking to correlate the course of milk secretion with the laws governing the rate of chemical reactions, have expressed the lactation curve in the form of an exponential equation, $M_t = M_o e^{-kt}$, in which M_t is the rate of yield at any time, t, and M_o is the initial rate of yield. This is the type of equation representing the course of a monomolecular reaction in which M_o would represent the initial amount of the substance undergoing such reaction and M_t would represent the amount of the substance remaining unchanged at any time, t. This similarity in form of the equations Brody *et al.* interpret in favor of a limiting substance governing the maximum rate of milk secretion at any stage of lactation, the limiting substance undergoing monomolecular change which inactivates it so far as its effect on the rate of milk secretion is concerned.

The above results are based on the raw data of milk yield. It has been shown by Gaines and Davidson[3] that the equation applies even more closely to the data of energy yield than it does to milk yield. While the equation is admirably adapted to describe the lactation

[1] Sturtevant, E. L., *Rep. New York (Geneva) Agric. Exp. Station*, 1886, 21–23.

[2] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1922–23, v, 441.

[3] Gaines, W. L., and Davidson, F. A., *J. Gen. Physiol.*, 1925–26, ix, 325.

curve in simple mathematical terms, the chemical interpretation is open to question. From the viewpoint of Brody *et al.* the factor k of the equation corresponds to the specific velocity constant of the chemical reaction. It is the purpose of the present paper to present some results showing the k values of individual lactation curves as bearing on the interpretation to be placed on the group behavior.[4]

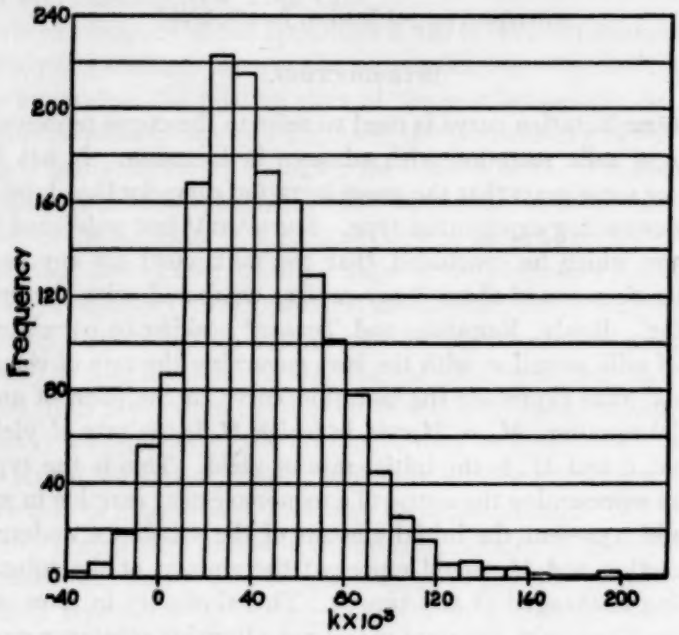

FIG. 1. Frequency distribution of 1534 Guernsey records with respect to rate of decrease per month (k) in rate of yield.

Individual Lactation Curves.

A curve of the type $\frac{dy}{dt} = Ae^{-kt}$ has been fitted by an adaptation of the method previously described to each of 1676 Guernsey records. In the equation y = yield and t = time in months. Yield for a month

[4] The data here given are taken from a manuscript presenting a broader investigation of the k values, which has been submitted for publication to the Agricultural Experiment Station.

represents the rate of yield at the middle of that month, with an entirely negligible error. Energy value of the milk solids had been used as being the most fundamental of the several available measures of yield. The distribution of the k values of these records, excluding 142 which were highly irregular, are given in Fig. 1. For a chemical interpretation of the lactation curve we may consider that the data of Fig. 1 represent 1534 determinations of the velocity constant k.

It will be noted from Fig. 1 that the k values fall into a quite regular order approaching a normal distribution. The mean of the array is .04425 ± .00055; standard deviation, .03219 ± .00089; and coefficient of variability, 72.75 ± .89. There is thus shown a high degree of variability in the k constants.

Ascending Lactation Curves.

It is to be noted further that 83 of the lactation curves, 5.41 per cent of the total, show negative k values, that is, the slope is positive. The velocity constant is not only highly variable, but apparently even reverses its sign in an appreciable number of cases. Obviously some modification of the simple monomolecular interpretation is necessary.

The group behavior of these ascending records is not in good conformity with the equation type, being somewhat aberrant at the start and finish. Indeed, to anyone conversant with the great energy transformations performed by the lactating cow it would be absurd to suppose that the lactation curve could continue to ascend for more than a limited time. The point that any hypothesis must satisfy is that within this group the curve does ascend for 9 or 10 months.

Irregular Lactation Curves.

Under this head are to be considered the 142 records not included in Fig. 1 and necessary to complete a representative sample of the Guernsey records. The average of this group of irregular records is given in Fig. 2 together with 4 individual curves to show the diversity of the records that enter into the average. If the monthly yields are plotted on a logarithmic scale against time on an arithmetic scale,

they should fall on a straight line to satisfy the equation. The data are plotted in Fig. 2 to show the degree of conformity to this relation.

The 142 records which we are now considering are so irregular that they were rejected from the main study on the ground that they must have been unduly influenced by extraneous factors. The point of interest in the present connection brought out in Fig. 2 is, that treated

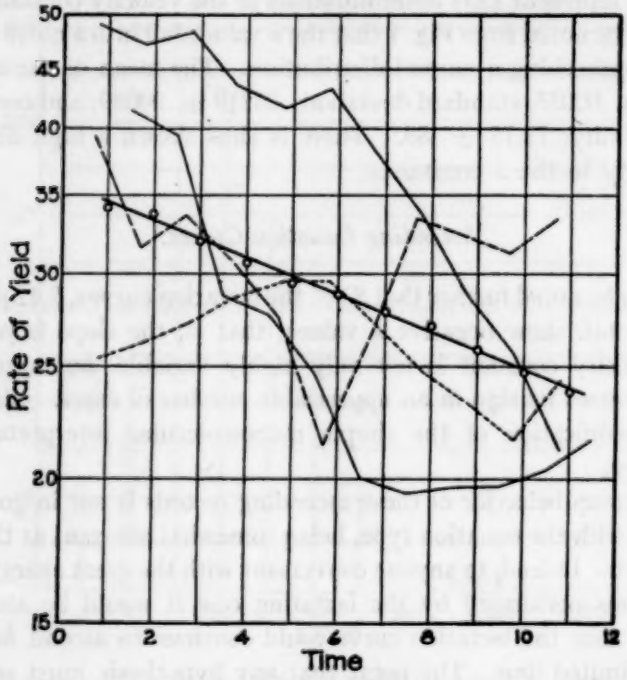

FIG. 2. Showing the average of 142 irregular lactation curves (circles) and 4 individual curves to indicate the diversity. Time is expressed in months from calving. Rate of yield is expressed in pounds of 4 per cent milk per day. One pound 4 per cent milk = 331 large calories.

as a group we get a result conforming acceptably with the equation. The meaning of the regularity of the group behavior is not clear, when the group is composed of such variable individual elements. It means that the individual deviations from the type curve are of a compensating nature. On the theory of a limiting substance determining the maximum rate of milk secretion, it is easy to account for minus deviations through the effect of other factors in preventing

realization of the maximum, but the difficulty lies in accounting for the plus deviations which serve to counterbalance the minus deviations.

Factors Affecting the Rate of Decrease.

That the k values of the lactation curve equations are greatly affected by conditions of feeding and management of the herd is sufficiently evident from the difference in the performance under advanced registry conditions and conditions of commercial milk production. It may be presumed that the same cows that show under advanced registry a mean k value of .044 would show under the less favorable commercial conditions a mean value of .09 to .1. Obviously nutritional conditions are a powerful factor affecting the rate of decrease.

The k values are also closely related to the initial rate of yield, the correlation between k and A being $r = .535 \pm .012$. On the limiting substance theory the initial quantity of this substance is directly proportional to A. The limiting substance is assumed to disappear in accordance with a monomolecular reaction. The velocity constant of the reaction must be assumed, therefore, to vary directly with the initial amount of the limiting substance, in order to satisfy the observed results. One of the laws of unimolecular reaction is that the velocity constant is the same regardless of the concentration of the reacting substance. Consequently, the observed relation between k and A is in conflict with the theory of a limiting substance and its monomolecular inactivation.

On the other hand, regarded as a nutritional matter and bearing in mind the large energy requirements of lactation, it is not surprising that the rate of decrease should be greater the greater the initial rate of yield.

SUMMARY.

The validity of the assumption of a substance determining the rate of milk secretion and undergoing monomolecular destruction, based on group behavior, is questioned on the evidence from a large number of individual lactation curves. It seems probable that the rate of decrease in the rate of milk secretion with advance in lactation is dependent upon factors of a nutritional nature.

RATE OF RESPIRATION AS RELATED TO AGE.*

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(Accepted for publication, June 21, 1926.)

It is usually stated in the literature that respiration is most rapid in the young and actively growing parts of a plant and decreases with age; and sometimes it is said that respiration conforms to the grand period of growth.

Considerable experimental evidence upholds the first statement. Bonnier and Mangin[1] studied the respiration of several different plants, among them *Evonymus japonica*. Leaves 1 year old respired more per volume of leaf than leaves produced the year of the experiment. Data for other plants were obtained (at different temperatures as far apart as 13 degrees but no corrections were made). M. A. Maige[2] found that most flowers produce more CO_2 per gm. of green weight in the bud stage than when open, but some respire most actively in the open stage. G. Maige[3] found that the intensity of respiration of floral organs decreased with age, except in the pistil, which often showed increased respiration as long as it continued to develop. Nicolas[4] found that of two samples of twigs the younger had a higher rate of respiration. Studying young and adult leaves he obtained the same results. Briggs, Kidd, and West[5] state that, "the evidence available (*Helianthus*) is that the respiration per unit dry-weight of the whole plant at constant temperature decreases with age."

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* Paper from the Department of Botany of the University of Michigan, No. 250.

[1] Bonnier, G., and Mangin, L., Recherches sur les variations de la respiration avec le developpement des plantes, *Ann. sc. nat. bot.*, 1885, ii, series 7, 315.

[2] Maige, M. A., Recherches sur la respiration de la fleur, *Rev. gén. bot.*, 1907, xix, 8.

[3] Maige, G., Recherches sur la respiration des différentes pièces florales, *Ann. sc. nat. bot.*, 1911, xiv, series 9, 1.

[4] Nicolas, G., The variation in the respiration of plants in proportion to age, *Bull. soc. hist. nat. Afrique nord*, 1910, No. 7, 109. (*Exp. Station Rec.*, 1912, xxvi, 628.)

[5] Briggs, G. E., Kidd, F., and West, C., A quantitative analysis of plant growth. Part II, *Ann. Applied Biol.*, 1920–21, vii, 202.

On the other hand A. Mayer,[6] and also Rischawi[7] claim to have demonstrated that the respiration follows the grand period of growth. These investigators enclosed seedling plants in a respiration chamber, and either by daily measurements of the O_2 intake or of the CO_2 given off arrived at the conclusion that there was a grand cycle of respiration conforming to the grand period of growth. In their experiments the normal photosynthetic activity of the plants was prevented and no account was taken of any changes in weight due to the metabolism of the plants. In other words, their results record the respiratory changes in a growing plant independent of any loss or increase in the weight of the plant during its growing period.

The purpose of the present investigation has been to determine carefully the relation between rate of respiration and age. To accomplish this an effort was made to determine the comparative rate of respiration in successive leaves of several plants. In the plants chosen new leaves continue to appear at the top of the plant until the panicles are formed. Before these are formed, however, some of the older leaves at the base have withered away. The successive leaves of these plants, therefore, constitute an age series. In corn the total leaves may number anywhere from 18 to 24, but at any one time there are usually not more than 8 to 12 leaves. In sorghum the total number of leaves developed is 25 to 30 with 8 to 15 active leaves present at one time. In oats and wheat there are only 4 to 6 leaves at any one time. The number of active leaves present appears to be related to age, varietal differences, and water supply. During a dry season the life cycle of a leaf is shorter than during a wet season.

In determining the rate of respiration, the active leaves of the plants were removed and the total CO_2 given off by each leaf was simultaneously determined, by placing the leaves in a battery of Pettenkofer tubes. Inasmuch as the leaves of a plant were all removed at the same time, placed in their respective respiration chambers at the same time, and the CO_2 given off was determined for the same interval, and further, since temperature and other external conditions were identical, it follows that any differences in the rate of respiration are due to differences in the leaves themselves.

[6] Mayer, A., Ueber den Verlauf der Atmung beim keimenden Weizen, *Landw. Versuchs-Stationen*, 1875, xviii, 245.

[7] Rischawi, L., Einige Versuche über die Atmung der Pflanzen, *Landw. Versuchs-Stationen*, 1876, xix, 321.

The rate of respiration given is the amount of CO_2 given off per gm. of dry matter or per gm. of green weight for the duration of the experiment. Since there were variations in the duration of the experiments and also in the temperature conditions, the rates of respiration in one experiment cannot, except in a general way, be compared with the rates in another. In general, the duration of an experiment was from 18 to 22 hours.

All possible precautions were taken to insure that the air entering the respiration chambers was free from CO_2; care was also taken that all the CO_2 was absorbed by the barium hydroxide tubes. The barium hydroxide after having been standardized was kept in containers entirely free from CO_2.

To illustrate the procedure a typical experiment will be described. A corn plant with ten healthy leaves has been selected in the field. It is brought into the laboratory and the leaves are carefully removed and placed in separate bottles of 500 cc., capacity which are completely covered with black paper. The experiment is arranged as follows: First come 10 $CaCl_2$ towers filled with pieces of soda lime to absorb the CO_2 from the entering air; following each tower is a bottle containing a solution of BaOH, to indicate whether all the CO_2 has been absorbed; to these bottles are attached the respiration chambers, covered with black paper, which in turn are attached to the Pettenkofer tubes, containing 100 to 150 cc. of standardized BaOH (the amount depends on the size of the plant; with large plants more BaOH is used than with small plants); each tube is followed by a bottle containing a solution of BaOH, to insure that all CO_2 has been absorbed by the standardized BaOH; these bottles are connected by Y-tubes to a single aspirator, by means of which the air is drawn through the apparatus. The rate and size of the gas bubbles passing through each tube are regulated so as to be the same in all tubes. Before the experiment is started the respiration bottles are thoroughly freed of all CO_2 by running CO_2-free air through them. The experiment is then run for 22 hours. At that time the BaOH in each tube is titrated with N/10 oxalic acid.

1 cc. of N/10 oxalic acid is equivalent to 0.0022 gm. of CO_2, and from the difference between the original titrations of the standardized BaOH and the titrations at the end of the experiment the amount of CO_2 can readily be calculated.

The amount of CO_2 has been calculated both on the basis of dry and of green weight. The results are similar, and as it is more usual to employ dry weight than green weight, and as the respiration is undoubtedly more closely related to the dry material than to the water of the plant, only the data calculated for dry weight are given. As pointed out before, no two experiments were carried out under identical conditions, nor were the plants themselves identical; some of these experiments were performed during the summer of 1923, others

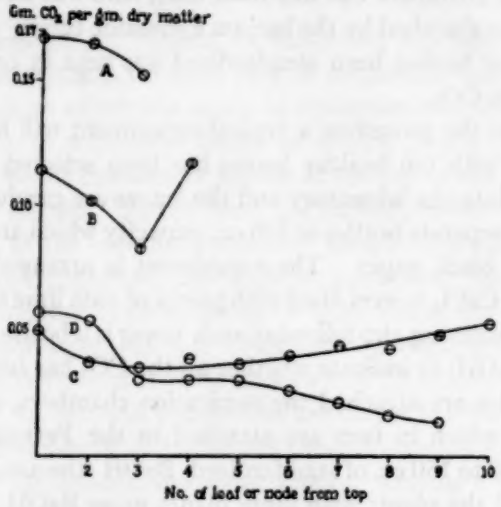


FIG. 1. Curve *A* represents the respiration of 3 corn plants about 4 inches high, with 3 leaves; *B*, 1 corn plant 6 inches high with 4 leaves; *C*, 1 corn plant at the time of pollination with 10 leaves. Curve *D* represents the respiration of a corn stem cut up in such a way that each piece contains one node and part of the contiguous internodes.

during 1924. For this reason no average of the experiments can be obtained. A few of the experiments have been selected as illustrating the condition in these plants.

Corn (*Zea Mays*) was the original plant worked with and the results of a few of the experiments are given in Fig. 1. Curves *A*, *B*, and *C* represent plants of different ages with varying number of leaves. Curve *A* shows that there is a decrease in rate of respiration with age when the plant is very young, while *B* shows that as the plant ages

and the number of leaves increases the rate of the older leaves increases above that of those slightly younger; and *C* (a plant with ten leaves) shows that the oldest leaves may actually respire more than do the youngest leaves on the plant. Curve *D* represents an experiment with the corn stem, which shows that there is a decrease in respiration with increase in age.

In Fig. 2 Curves *A*, *B*, and *C* represent respiration in *Sorghum vulgare*. All of these experiments show that at first there is a decrease in respiration as the leaves age, but that after a certain age has been

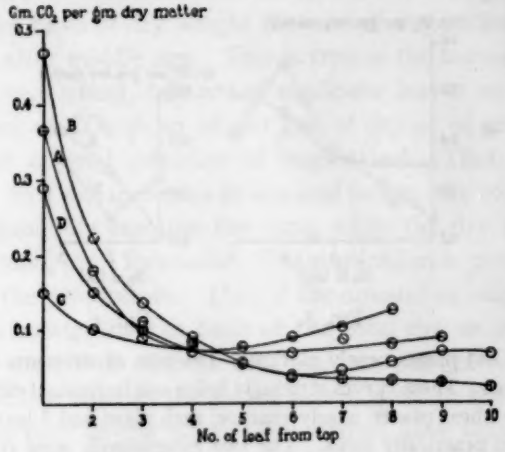


FIG. 2. Curve *A* represents the respiration of 4 sorghum plants 1 foot high with 8 leaves; Curve *B*, 1 sorghum plant 3 feet high with 10 leaves; *C*, 1 sorghum plant developing tassel, with 9 leaves; Curve *D*, sunflower plant about half grown, with 10 leaves.

reached there is a gradual increase in rate, which in some instances is nearly as great as that in the youngest leaves. Curve *D* represents leaves of sunflower (*Helianthus annuus*), and in this as well as in other experiments on sunflower there is a decrease from the first with no subsequent increase.

Fig. 3 represents results obtained with oats (*Avena sativa*). The two experiments were conducted at the same time. These experiments show that the respiration in the oldest leaves is much more rapid than in the youngest.

In Fig. 4 are results of two experiments with nearly mature wheat (*Triticum sativum*). These plants had five leaves but the two oldest ones were dying and so cannot be counted. Both experiments show that the oldest healthy leaves respired more vigorously than the youngest leaves of the plants.

The plants represented in Figs. 3 and 4 were nearly mature and the youngest leaves had already reached the age at which the respiration is at its lowest; all the curves but *B* of Fig. 4 show only increase in respiration with age rather than a decrease followed by an increase.

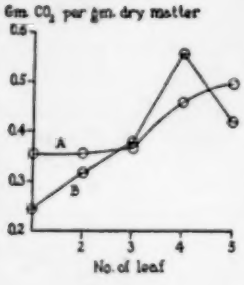


FIG. 3.

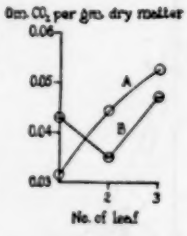


FIG. 4.

FIG. 3. Two oat plants nearly mature. The two experiments were conducted at the same time. These curves strikingly bring out individual differences.

FIG. 4. Two wheat plants, nearly mature; each plant had 5 leaves, but the two lowermost were practically dead. The two experiments were conducted at the same time. They also show individual differences.

It is quite apparent from the data given that when dry weight is taken as a criterion for judging the comparative rate of respiration in the various leaves of corn, sorghum, sunflower, oats, and wheat the respiration cycle does not correspond to the grand period of growth, nor is there a decrease in respiration with age except in the leaves of sunflower and corn stem. The reason other investigators have not noted this before is that not enough controlled experiments have been performed. In most instances a few leaves of one age were taken at one time at a certain temperature and a few leaves of a different age at another time, at a temperature different from the first, without any correction being applied. As far as the writers are aware no experiments have been performed in which a series of leaves or other plant

parts, differing in age, have been studied at the same time. It is obvious from the figures given in this paper that, if only two leaves had been taken, in practically all instances the older leaves would have respired less than the very youngest on the plant, but it is equally obvious that by taking very young leaves, middle aged, and old leaves at the same time, the old leaves are found to respire more rapidly than the middle aged ones, though usually less than the youngest.

The writers are not prepared to say that as protoplasm ages it respires less and that as it gets still older it begins to respire more actively. But it seems that when the amount of CO_2 given off is calculated on the basis of dry weight the rate of respiration increases to some extent after middle age. This is true of the leaves of corn, sorghum, oats, and wheat, but not of sunflower leaves and corn stems.

The amount of CO_2 given off per gm. of dry or of green weight is probably not a good criterion of respiration. That, however, is customary. As a cell increases in size and in age, the total amount of protoplasm probably remains the same while the dry material (cell wall, stored food, etc.) increases. The respiration is presumably connected with the protoplasm. Then if the amount of respiration of an old leaf is calculated on the basis of the total dry weight (cell wall, stored food, etc.) it is obvious that the rate per gm. of dry weight is going to be less than in the young leaves, though the rate per gm. of protoplasm may be the same. The writers have unpublished data to show that as leaves grow older there is an increase in the percentage of dry material up to a certain age, when there is sometimes a decrease. A method which would take into account only the amount of protoplasm would be much more accurate. Perhaps the CO_2 could be calculated on the basis of amino nitrogen or total nitrogen in a plant part.

SUMMARY.

In the present paper it is shown that as the leaves of corn, sorghum, wheat, and oats increase in age there is a decrease in rate of respiration; but that as the leaves become still older (past about middle age) the rate gradually increases.

CONCERNING THE INFLUENCE OF POLARIZED LIGHT ON THE GROWTH OF SEEDLINGS.

By DAVID I. MACHT.

(From the Pharmacological Research Laboratory, Hynson, Westcott, and Dunning, Baltimore.)

(Accepted for publication, June 11, 1926.)

The biological effects of polarized light have, until a year or two ago, not been the subject of any scientific experimental study. Recently a few contributions on the subject have appeared. Baly and Semmens published a short note describing the stimulating effect of polarized light on the hydrolysis of starch by diastase (1). These observations were confirmed by the present author (2) who at the same time published several short communications on the pharmacological and other biological effects of polarized light (3) and also described in collaboration with Justina Hill some experiments on the growth of yeast and bacteria (4). In this last communication, Miss Hill and the author called attention to the apparent stimulation of bacterial growth by polarized light. Similar observations on bacteria were published independently by Morrison (5), and recently two Indian investigators have also published a short note claiming that the bacteria of typhoid fever and cholera thrive better in polarized light than in non-polarized light (6). In the present paper the author proposes to describe a series of experiments on the growth of seedlings in polarized and non-polarized light which were begun early in 1924 and continued up to the present time.

Method.

The growth of young seedlings of *Lupinus albus* in a nutrient phytophysiological (Shive's (7)) solution was followed by measuring the elongation of the roots at intervals of 24 hours. The influence of polarized and non-polarized light was studied by means of the following apparatus which was designed jointly by the author and Professor

A. H. Pfund of the Department of Physics of the Johns Hopkins University and was constructed under Professor Pfund's supervision and calibrated by him. A box or cell in the form of a truncated pyramid was constructed 80 cm. high, with a lower base 60 × 45 cm. and upper end about 25 cm. square, the back of the wall of the cell being perpendicular to the base, and the front wall and *door* being slanting. At the upper or small end of the cell a socket is fixed into which is inserted a large round Mazda tungsten nitrogen electric bulb, of 500 watts power, which serves as a source of light. The lower part of the apparatus or cell is divided into two compartments, completely separated from each other by a blackened partition. The light of the Mazda lamp is allowed to penetrate into the chamber on one side of the apparatus after first passing through a dozen plates of smooth glass, placed at the "polarizing" angle, so that this chamber is illuminated with highly polarized light. The light from the same Mazda lamp, on the other hand, is allowed to penetrate into the second or neighboring chamber after first passing through a pile of smooth plates of glass placed *perpendicular* to the line of propagation of the light, so that this second chamber is illuminated with non-polarized light. The number of glass plates in this second pile was adjusted so that the intensity of the non-polarized light was just equal to the intensity of the polarized light in the first chamber. By boring apertures in the floors of the two respective chambers and taking spectrophotographs of the two transmitted lights, it was found that the spectral range of light waves in the polarizing and non-polarizing chambers was the same, the shortest waves transmitted being about 3650 Ångstrom units. The temperatures in the two chambers were nearly the same, not deviating from each other more than 0.5°C.

The source of light was an electric bulb of 700 candle power. This intensity was of course cut down by passage through the piles of plates, but the intensity of the transmitted light in each chamber was made the same by photometric calibration in the Physics Laboratory, performed by Professor A. H. Pfund. The intensities in the two chambers were compared by Professor Pfund by reflecting the lights passing through the two sets of glass plates, from a white surface, and allowing the rays to pass through a Lummer tube. The light from the two chambers was thus reflected *diffusely*, and hence was *depolarized*

FIG. 1.

FIG. 2.

FIG. 1. Polarizing apparatus. Front view with door removed.

FIG. 2. Polarizing apparatus. Side view. Right wall removed and showing the chamber illuminated with polarized light.

before the comparison was made. Thus while the eye was used in comparing, it could not be argued that there might be a difference in the physiological effects of polarized and non-polarized lights on the eye. Of course such an objection would be purely hypothetical, as, so far as is known, no difference in the effects on the eye between polarized and non-polarized lights has ever been noted, and if such a difference should be experimentally demonstrated, it would be a fundamental physiological discovery.

In order to make sure that small variations in intensity of the control did not affect the results, a number of experiments were made with the non-polarized light of a slightly greater or slightly lesser intensity than the polarized light (by changing the number of plates in the control chamber). Such variations did not appreciably change the marked effect of the polarized light.[1]

The temperatures in the two chambers were the same to within a fraction of a degree, as indicated by thermometer readings and also by thermographic tracings. Here again a number of experiments were made, in which the temperature in the control chamber was purposely made a little higher or a little lower than in the polarized chamber, respectively, and the results obtained still showed a definite stimulation of growth produced evidently by polarized light.

RESULTS.

Two sets of *Lupinus* seedlings, A and B, generally of 10 each, were carefully measured, then placed in hard glass tubes with Shive's solution, and one set was put in each chamber, Set A in polarized light, and Set B in non-polarized light. The plants were irradiated by the polarized and non-polarized lights, during the daytime, and were left in the dark overnight, when the electric current in the laboratory was turned off. On measuring the growth of the roots of the two sets of plants on the following day it was found that the seedlings exposed to polarized light had grown distinctly more than the other set. The two sets of plants were then interchanged, by placing them in the opposite chambers, that is Set B in polarized light and Set A in non-

[1] In other experiments with polarized light, to be published later, intensities were compared by means of a bolometer.

polarized light, and exposed to the two kinds of light again. On the following day, when the growth of the roots was again measured, it was found that the original "non-polarized" set (B) which was this time exposed to polarized light actually outgrew the original "polarized" set (A) of plants, which, on this second day, was placed in the non-polarizing chamber.

The two sets of plants were again reversed for a second time, and it was found again that the seedlings grew more in polarized light. On reversing the position of the two sets a third time, better growth in polarized light was again observed. Such experiments with *Lupinus* seedlings were made a number of times with the same results. The following protocols will serve as illustrations.

TABLE I.

Set A.					Set B.				
Seedling No.	Normal length.	Polarized light.	Non-polarized light.	Polarized light.	Seedling No.	Normal length.	Non-polarized light.	Polarized light.	Non-polarized light.
	mm.	mm.	mm.	mm.		mm.	mm.	mm.	mm.
1	38	49	60	88	1	37	46	57	68
2	35	46	53	80	2	31	50	69	80
3	31	53	60	82	3	23	46	57	64
4	33	39	48	65	4	31	47	62	70
5	24	49	56	71	5	27	43	62	70
6	24	60	64	70	6	34	52	59	64
7	25	50	62	71	7	27	44	70	75
8	29	51	56	64	8	33	47	48	78
9	34	44	56	77	9	33	50	68	77
10	27	41	49	72	10	30	37	65	69
	300	482	564	740		306	462	617	715
		Increment 182.	Increment 82.	Increment 176.			Increment 156.	Increment 155.	Increment 98.

Table I gives the results of an experiment made on 2 sets of plants of 10 seedlings each. Set A was exposed to polarized light on the 1st day, to non-polarized light on the 2nd day, and to polarized light again on the 3rd day. In the case of Set B the order of exposure was

reversed, that is on the 1st day the plants were exposed to non-polarized light, on the 2nd day to polarized light, and on the 3rd day again to non-polarized light. The normal or original length of each root is indicated in the first column and the length at the end of each 24 hours in the successive columns. It will be seen that the increment in every case was greater when the seedlings were exposed to polarized

TABLE II.*

	Set A.						Set B.				
No.	Apr. 26. Original length.	Apr. 27. Polarized.	Apr. 28. Non-polarized.	Apr. 29. Polarized.	Apr. 30. Non-polarized.	No.	Apr. 26. Original length.	Apr. 27. Non-polarized.	Apr. 28. Polarized.	Apr. 29. Non-polarized.	Apr. 30. Polarized.
	mm.	mm.	mm.	mm.	mm.		mm.	mm.	mm.	mm.	mm.
1	47	55	69	81	76	1	33	57	70	82	72
2	42	60	78	74	79	2	32	58	61	68	82
3	47	53	65	87	70	3	36	51	68	70	70
4	38	66	69	71	75	4	45	53	75	78	71
5	48	50	62	76	80	5	42	68	74	79	77
6	32	64	64	77	76	6	40	57	71	81	74
7	39	52	65	77	70	7	45	56	71	72	64
8	45	67	62	77	85	8	36	64	71	76	76
9	37	48	69	80	71	9	49	53	64	69	80
10	35	54	68	68	72	10	42	62	76	73	75
11	36	68	66	72	72	11	48	49	61	77	78
12	42	59	68	73	70	12	44	47	60	78	81
13	37	59	74	74	72	13	33	61	73	77	79
14	51	63	63	77	75	14	48	60	69	75	72
15	42	56	58	73	69	15	45	57	63	75	85
	618	874	1000	1237	1112		618	852	1027	1130	1136
		Increment 256.	Increment 382.	Increment 619.	Increment 494.			Increment 234.	Increment 409.	Increment 512.	Increment 518.

* In a few cases measurement of seedlings shows a shrinkage on the last day.

light and more than that, when the two sets of seedlings were reversed in respect to the form of radiation, the same phenomenon was noted, namely, the greater growth in polarized light.

In the experiments summarized in Table II two other sets of plants consisting of 15 seedlings each were treated as above, only in this experiment the interchange from polarized to non-polarized light and

TABLE III.

Experiment 10.

Series A^1.			Series B^1.		
May 3, 1926.	May 4, 1926.	May 5, 1926.	May 3, 1926.	May 4, 1926.	May 5, 1926.
Normal.	Polarized.	Non-polarized.	Normal.	Non-polarized.	Polarized.
50.1 mm.	67.3 mm.	75.4 mm.	51.9 mm.	64.6 mm.	77.4 mm.
Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.	Mean temp. 26°C.

TABLE IV.

Experiment 11.

Series A^2. (Covered seeds.)			Series B^2. (Covered seeds.)		
May 3, 1926.	May 4, 1926.	May 5, 1926.	May 3, 1926.	May 4, 1926.	May 5, 1926.
Normal.	Polarized.	Polarized.	Normal.	Non-polarized.	Non-polarized.
30.0 mm.	35.7 mm.	39.1 mm.	29.6 mm.	35.3 mm.	37.8 mm.
Mean temp. 22°C.	Seeds covered. Mean temp. 22°C.	Seeds uncovered. Mean temp. 22°C.	Mean temp. 22°C.	Seeds covered. Mean temp. 22°C.	Seeds uncovered. Mean temp. 22°C.

TABLE V.

Experiment 12.

Series A^3. (Covered roots.)			Series B^3. (Covered roots.)		
May 3, 1926.	May 4, 1926.	May 5, 1926.	May 3, 1926.	May 4, 1926.	May 5, 1926.
Normal.	Polarized.	Non-polarized.	Normal.	Non-polarized.	Polarized.
27.2 mm.	36.4 mm.	40.0 mm.	26.4 mm.	34.2 mm.	41.3 mm.
Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.	Mean temp. 22°C.

vice versa was made on 4 successive days until the plants were too long to be measured conveniently. Here again it will be seen that in every case greater growth took place in polarized light. (In this table the order of the seedlings is not the same in each column as they were not placed in individual tubes but in flasks containing several seedlings each.)

Table III shows an analysis of the above phenomena. In Experiment 10, two sets of plants consisting of 10 seedlings each were placed

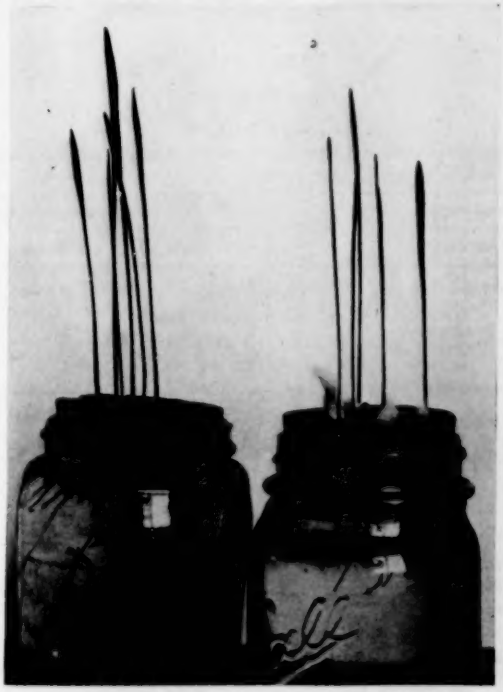
TABLE VI.

Squash Seedlings.

Set A.				Set B.			
No.	Original length.	Polarized 24 hrs.	Polarized 72 hrs.	No.	Original length.	Non-polarized 24 hrs.	Non-polarized 72 hrs.
	mm.	mm.	mm.		mm.	mm.	mm.
1	47	58	69	1	45	55	57
2	36	47	54	2	34	37	47
3	32	44	58	3	32	34	47
4	21	27	46	4	22	25	31
5	41	51	71	5	43	54	45
6	31	40	63	6	33	44	80
7	42	47	56	7	37	40	83
8	21	29	52	8	22	35	63
9	19	28	66	9	21	26	34
10	16	26	55	10	18	30	38(?)
	306	397	590		307	380	525
		Increment 91.	Increment 284.			Increment 73.	Increment 218.

in polarized and non-polarized light, the figures here given indicating the sum of the root lengths. It will be noted here again that each set of plants grew better in polarized light. Experiment 11 illustrates the effect of wrapping the seeds or cotyledonous portion of the plants with tinfoil. It will be noted that when the beans were wrapped in tinfoil and thus protected from light altogether, growth was exactly the same in both chambers. When, however, these wrappings were removed on May 5th, better growth occurred in polarized light than in non-polarized light. Experiment 12 shows the results obtained

when the stems and roots of the seeds were protected from the light but the seed portion was left uncovered. Here better growth took place in polarized light than in non-polarized light thus indicating that the stimulation of growth by polarized light is due to photochemical changes induced in the seed portion. This, of course, harmonized prettily with the findings of Baly and Semmens and the



Polarized. Non-polarized.

FIG. 3. Wheat seedlings.

present author in connection with the effect of polarized light on the diastatic hydrolysis of starch.

The author performed most of his experiments on seedlings of *Lupinus albus*. A number of experiments, however, were also performed on other plants. In Table VI are the results obtained with squash seedlings. Here again it will be noted that better growth

occurred in polarized light than in non-polarized light. A number of experiments were made with wheat seedlings; as these seedlings grow several roots which are difficult to measure, the growth was studied by measuring the elongation of the stems and leaves. In Table VII and Fig. 3 are shown the results obtained in one such experiment. Two sets of 6 seedlings each were exposed to polarized light and non-polarized light and the length from the root to the tip of the blade of

TABLE VII.

Wheat Seedlings.

(See Fig. 3.)

Set A.	Set B.
In polarized light.	In non-polarized light.
6 seedlings.	6 seedlings.
Original length from roots to tip of blade, 402 mm.	Original length from roots to tip of blade, 406 mm.
3 days later, 656 mm.	3 days later, 588 mm.
4 " " 921 "	4 " " 804 "

TABLE VIII.

Helianthus (Sunflower).

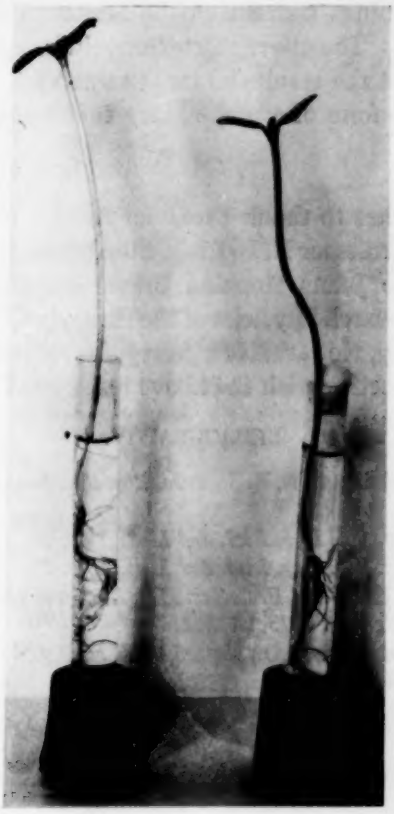
(See Fig. 4.)

Seedling A.	Seedling B.
Dec. 16, 1925. Length of stem, 120 mm.	Dec. 16, 1925. Length of stem, 120 mm.
Placed in polarized light.	Placed in non-polarized light.
Dec. 18, 1925. Length of stem, 140 mm.	Dec. 18, 1925. Length of stem, 125 mm.
" 19, " " " " 147 "	" 19, " " " " 127 "

each seedling was measured. It will be noted that better growth took place in polarized light. In several other experiments with wheat seedlings the growth of the plants was studied by weighing them. In this way it was also found that the seedlings exposed to polarized light weighed distinctly more than those exposed to non-polarized light. In Table VIII and Fig. 4 is shown the effect of polarized light on two seedlings of the sunflower, the length of the stems was measured and it will be noted better growth took place in the polarizing chamber.

SUMMARY.

While these experiments are not exhaustive, a sufficient number have been made to warrant the statement that the effect of polarized light of the visible spectrum on the growth of various seedlings and



Polarized. Non-polarized.

FIG. 4. Sunflower seedlings.

more particularly on the growth of *Lupinus albus* is somewhat different from that of non-polarized light. This is especially convincing in view of the results obtained with double sets of plants which were alternately exposed to polarized and non-polarized lights of the same

intensities and at the same temperature. In every experiment thus performed the set which was placed in a polarizing chamber grew better. It is, furthermore, interesting to note that the phenomenon above observed did not take place when the seed portion of the plants was protected from light by wrapping with tinfoil. This agrees well with previous findings concerning the action of diastase on starch in polarized light. The above researches will be continued on a more elaborate scale but the results so far obtained are deemed worthy of publication in the form of a preliminary communication at the present time.

The author wishes to thank Professor A. H. Pfund of the Physics Laboratory and Professor B. E. Livingston of the Laboratory of Plant Physiology of the Johns Hopkins University and Dr. William T. Anderson, Jr., Research Physicist of the Hanovia Chemical and Manufacturing Company, Newark, New Jersey, for valuable suggestions and criticisms in connection with the above research.

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ON CURVES OF GROWTH, ESPECIALLY IN RELATION TO TEMPERATURE.

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I.

Growth generally manifests an accelerated velocity during the mid-portion of a developmental cycle, so that the curve of bulk with time is sigmoid. This fact gave rise (Ostwald, 1902; Errera, 1899–1900; Robertson, 1907–08) to the view that the accumulation of material during growth may be described by the equation for a first-order process in which one of the products of the transformation acts as catalyst. This conception has had a vigorous enlargement (Robertson, 1923), looking toward the description of the velocity of development as governed by a succession of first-order chemical processes, "master reactions," which are self-accelerated.

Several difficulties are connected with the application of this idea. One of these is that in fitting simple logistic (autocatalytic) curves to actual data, it has usually been assumed (*cf.* Robertson, 1923; 1925–26) that the curve of growth in any cycle is symmetrical about a mid-point of inflection. This follows from the nature of the equation commonly taken to give the course of such an autocatalyzed process:

$$\frac{dx}{dt} = K\,x\,(A - x), \tag{1}$$

where A signifies the initial endowment of the growth-promoting precursor, x the amount formed after time t. The point of inflection in the integral curve of this equation is located at

$$x = A/2.$$

A method of testing the sufficiency of this form of the autocatalysis equation is afforded by the study of rates of development as controlled

by temperature. The autocatakinetic relationship (Lotka, 1925) is of so very general a character, arising in any kind of a situation where a limited progress is facilitated by the conditions created through its initiation[1] but progressively inhibited by proportionately enforced exhaustion, that some test of this sort is necessary if the form of growth curves is to be satisfactorily understood. Thus if the simple logistic were an adequate description of growth velocity, temporary alteration of the temperature of the development would not be expected to modify the temperature characteristic (Crozier, 1924–25, *a*) for the remainder of the development. Since the temperature characteristic for the velocity constant K must be constant, changing the temperature therefore merely multiplies the time coordinates of the growth curve by a constant. The temperature characteristics obtained from what may be termed "partial developmental periods" should be the same as for the total developmental interval (within one cycle). There is adequate indication (Bliss, 1925–26) that this may not be the case. Consideration of this fact results in a modification of the formulation of the "autocatalytic" curve of growth. The modification has the merit of greater consonance with chemical theory, and of indicating a direct interpretation of the sort of results to be expected when the temperature is changed during the course of a developmental cycle. It may also give some light upon the nature of temperature characteristics for development at constant temperatures.

Let it be supposed that at the beginning of a developmental cycle there is available an unrenewable quantity, A, of a substance giving rise to another, x, which determines the velocity of growth. We are especially interested in "velocities of growth" as measured by the reciprocals of the times required to attain a given stage of development. We will suppose that the material A gives rise to x by a first order reaction, and that x serves as catalyst for this change. The reaction $A \rightarrow x$ will therefore be governed by a velocity constant (K_1) proper

[1] An interesting instance is given by the growth of knowledge of the variety of the amino acids, as plotted by Cohn (1925). A still different type of S-shaped curve may result from estimations of growth in which the numbers of cells of colonial protozoans are counted (Fauré-Fremiet, 1922); in certain species the mode of dichotomy results in the curve for number of individuals against time appearing "autocatalytic."

to it in the absence of the influence of x, and also by the velocity constant due to catalysis by x. The decomposition of A must therefore be conceived as made up of two parallel reactions, and its differential equation is then

$$\frac{dx}{dt} = (K_1 + K_2 x)(A - x), \tag{2}$$

where K_2 is the velocity constant associated with x as catalyst.

The velocity of formation of x will pass through a maximum when

$$x = \frac{K_2 A - K_1}{2 K_2}. \tag{3}$$

Therefore, if any change of condition, such as temperature, influences K_2 and K_1 unequally, the form of the curve connecting x with time will be changed and the point of inflection will move to a new relative position. Thus when K_2 is made relatively smaller, the inflection point occurs earlier (assuming A the same), and the shape of the curve is significantly altered.[2]

It is to be noticed that in such a system the point of inflection is found at $x = A/2$ when K_1 is of inappreciable magnitude; whereas, in case K_2 is very small the equation approaches that of the usual monomolecular curve without detectable autocatalysis.

II.

Integration of (2) yields

$$t = \frac{1}{K_1 + K_2 A} \ln \frac{A (K_2 x + K_1)}{K_1 (A - x)} \tag{3}$$

The curve of this equation may be applied to various series of observa-

[2] This formulation seems simpler, and leads to more suggestive consequences so far as concerns the planning of experiments, than does Robertson's (1923) suggestion of the pseudo-reversible character of the "master process" in growth; moreover, it would appear to be the obviously correct equation for an autocatalytic system. Robertson pointed out that if a reverse process in the growth equation is of higher order than the direct, the curve of increasing bulk with time becomes asymmetric about the inflection point; many if not most growth-cycles are in fact asymmetric (*cf.* Brody, 1925–26). A different mode of dealing with the asymmetry has more recently been employed by Robertson (1925–26), which amounts to assuming that x has a positive value at the beginning of a cycle.

tions; for example, to the growth in height of sunflower plants (data of Reed and Holland, 1919). The fit (Fig. 1) may be made distinctly better than when the "simple" curve of autocatalysis is employed. An adequate test of the descriptive validity of this equation for growth might be sought in the curve of increasing weight for a mammal, where temperature variation may be neglected. Donaldson's data on

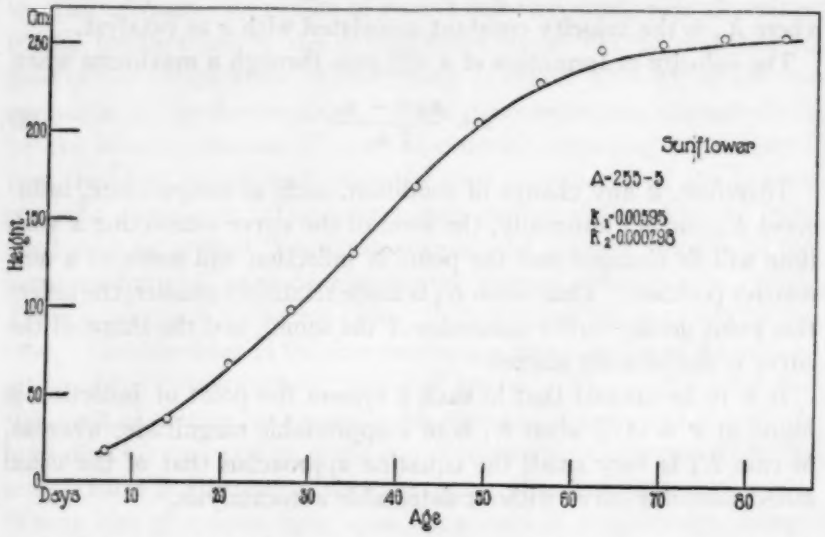

FIG. 1. Growth in height of the sunflower; data of Reed and Holland (1919); the curve is that of the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A\,(k_2 x + k_1)}{k_1\,(A - x)},$$

the cycle starting at $x = 5$ cm., with $k_1 = 0.00595$, $k_2 = 0.000298$; A, the mature height, is taken = 250.

the growth of the rat (Donaldson, 1915) are suitable for such a test, though undoubtedly complicated by the cyclic character of growth in mammals (Donaldson, 1906; Robertson, 1907–08; Brody and Ragsdale, 1922–23). Robertson (1923) has fitted to this data two "fused" logistic cycles, but the agreement is not especially good. In Fig. 2 it is shown that these data are fitted sufficiently well by the autocatalytic equation in the form $dx/dt = (K_1 + K_2 x)\,(A - x)$, except that for a

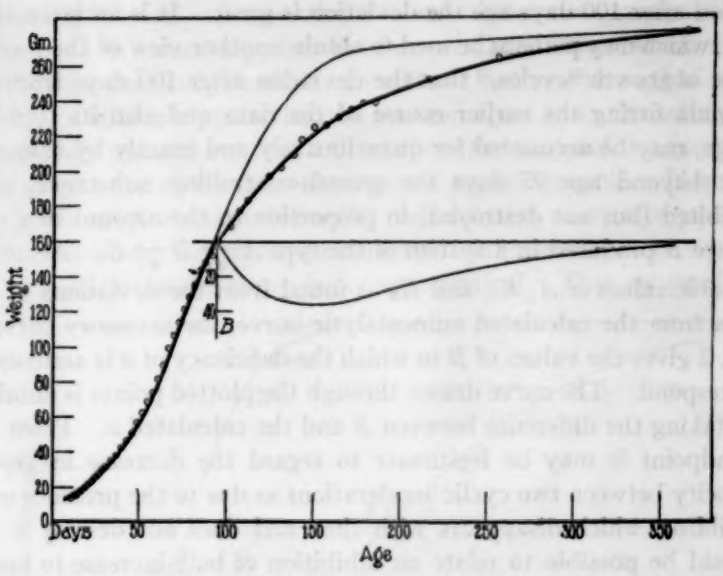

FIG. 2. Data on the growth of unmated male white rats (Donaldson, 1915) adhere fairly well to the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A (k_2 x + k_1)}{k_1 (A - x)}$$

for the first 100 days. It is assumed that the weight reflects the amount of a growth-determining material x, produced in a reaction $A \rightarrow x$, which is catalyzed by x; that the value of A is given by the maximum weight (for convenience, 280 gm.); and that the cycle starts at $x = 10$ gm. The constants for the curve as drawn are $k_1 = 0.00135$, $k_2 = 0.000135$; x = weight -10.

Beyond 95 days age, it may be supposed that the material x is inhibited by another, B, produced in a system of the type $M \underset{K_3}{\rightarrow} B \underset{K_4}{\rightarrow} N$, where M is unrenewable. The thin inverted curve is the curve of this process with $M_0 = 121$, $k_3 = 0.01663$, $k_4 = 0.02375$; the equation is

$$B = M_0 \frac{k_3}{k_4 - k_3} (e^{-k_3 t'} - e^{-k_4 t'}),$$

t' being counted from $t = 95$.

The curve drawn through the observed weights (circles) is obtained by subtracting B from x.

period after 100 days age the deviation is great. It is an interesting fact, which may perhaps be used to obtain another view of the mechanism of growth "cycles," that the deviation after 100 days from the formula fitting the earlier course of the data and also its terminal range, may be accounted for quantitatively and exactly by assuming that beyond age 95 days the growth-controlling substance, x, is inhibited (but not destroyed) in proportion to the amount of a substance B produced in a system of the type $A \xrightarrow[K_3]{} B \xrightarrow[K_4]{} C$. Assuming suitable values of A, K_3, and K_4 as found from the deviations of the data from the calculated autocatalytic curve, the accessory curve in Fig. 2 gives the values of B to which the deficiency of x is assumed to correspond. The curve drawn through the plotted points is obtained by taking the difference between B and the calculated x. From this standpoint it may be legitimate to regard the decrease in growth velocity between two cyclic accelerations as due to the presence of an inhibitor, which disappears with time and does not destroy x. It should be possible to relate an inhibition of bulk-increase to known physiological events in the organism dealt with. For the rat it can be pointed out that the maximum in the "correction curve" (B) occurs at an age of 150 days, at which time (Donaldson, 1924) the activity of the thyroid seems to attain a definite maximum and then to decline. This implies that thyroid activity is to be taken as hindering growth in bulk (at this age). Miss King's measurements of growth of rats (Donaldson, 1924) may be fitted in a similar way, with the maximum of the "correcting curve" at very nearly the same age. There is indication that the peculiar growth curve of man (summary of data in Davenport, 1926) may be accounted for in a similar way, with the maximum growth inhibition (male) at about 14 years; but the deviation from calculated autocatalytic curves is so extensive as to make difficult the adjustment of a "correction curve."

III.

The way in which the curve of equation (3) may be modified by changing the temperature is illustrated in Figs. 3 and 4. We may assume that for the completion of a developmental stage, say an instar or other definite interval in the differentiation of an insect, there must occur the production of a definite amount of a substance,

x, arising in a first order reaction $A \rightarrow x$, and that x is a catalyst for the reaction. We must suppose that in general the temperature characteristic for the catalytic effect of x will not be the same as that for the catalytic influence promoting the reaction apart from the action of x. As already pointed out, this will so affect the shape of the curve that the graphs at two temperatures are not superimposable. If one-half of the developmental period under consideration were to be passed at t°C., this would not mean that one-half of the development would be completed, since the curve is not symmetrical. Then on passing to

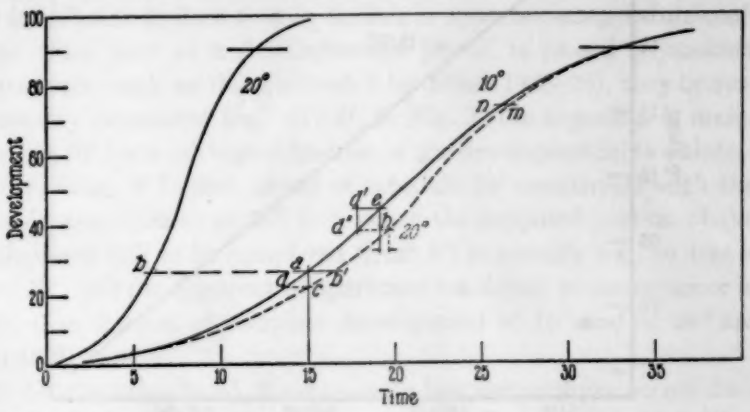

FIG. 3. Curves of the equation

$$t = \frac{1}{k_1 + k_2 A} \ln \frac{A(k_2 x + k_1)}{k_1 (A - x)},$$

with $A = 100$ in each case. (Time is in arbitrary units.)

At 20°, k_1 is assumed = 0.010
k_2 " " = 0.005
At 10°, k_1 " " = 0.005 (*i.e.*, $Q_{10} = 2$)
k_2 " " = 0.0017 (*i.e.*, $Q_{10} = 2.94$)

These curves are not superimposable; the dashed curve is that for the 20° conditions, expanded by multiplication of the abscissæ by a factor which makes the curves coincident at $x = 0.90A$.

If such curves are assumed to underlie development, and if we assume that at completion of a given stage x = a definite fraction of A, say = 90 per cent, then Q_{10} for velocity of development is (from the curves) 32.92 ÷ 12.02 = 2.74. As A is assumed larger and larger, the Q_{10} ratio for velocity of development approaches nearer and nearer to the Q_{10} for k_2. (If the assignment of Q_{10}'s is reversed, Q_{10} for $x = 0.90A$ is 2.13.) Additional details are discussed in the text.

another temperature, the development still to be completed would be (depending on the alteration in the ratio K_1/K_2) either more or less than the fraction uncompleted before the transfer; and the time required to produce the necessary fixed amount of x would be correspondingly greater or smaller than what would be calculated; hence the apparent temperature coefficient for the velocity of the partial development would not be the same as for the total development when passed at constant temperatures. (No account is here taken of differences produced by "breaks" in the curve of developmental rate with temperature.)

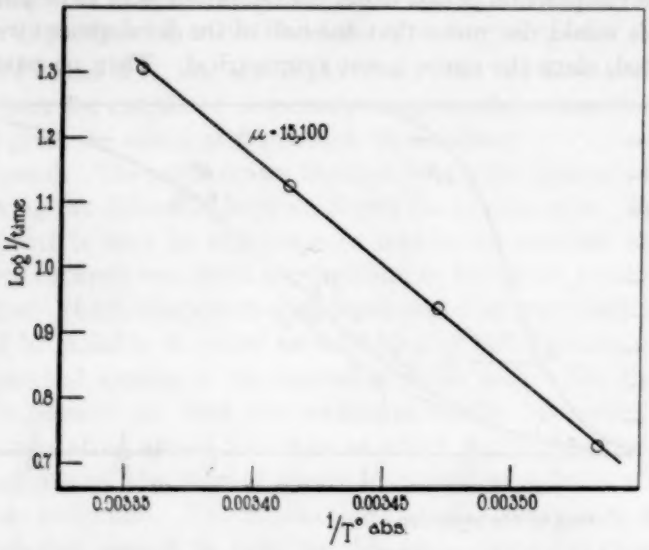

FIG. 4. From the autocatalytic curve, with assumed values $A = 500$, $k_1 = 0.08$, $k_2 = 0.0015$, and with μ for k_1 = 11,000, for k_2 = 16,000, values of the time are calculated to give $x = 490$. The reciprocals of these times are plotted logarithmically against $1/T°$ abs., at 10°, 15°, 20°, 25°C. The relationship is almost exactly rectilinear, and the temperature characteristic obtained from the graph (fitted line) is μ = 15,100. This shows that in a system of the sort discussed in the text it is possible to estimate an apparent critical increment which agrees fairly closely with that for one of two catalytic velocity constants concerned in an autocatalytic activity.

The assumption of a definite amount of x as marking the termina-

tion of a developmental interval is required because the end-product of growth is the same at each temperature. It is convenient to regard this amount as a fraction of A, the original endowment. With arthropods in particular, the succession of developmental intervals is so definite that one is forced to think of their termination as controlled by "trigger effects," such as might be provided through the accumulation of a governing substance to a critical concentration rather than by the asymptotic approach to completion which would correspond to the extinction of A in our model.

It is desirable to show that deviations in apparent temperature coefficient when part of a developmental period is passed at another temperature, such as those recorded by Bliss (1925–26), may be systematically accounted for. (1) If, in Fig. 3, the organism is maintained at 10° for a calculated fraction of the developmental time interval (*i.e.* to *a*) it is then ahead of schedule by comparison with the curve of development at 20° (b'); hence the supposed portion of the development still to be completed (from b') is actually less, on transfer to 20°, and the apparent temperature coefficient in consequence is larger than if rates of complete development at 10° and at 20° are compared.

(2) On the other hand, if exposure to low temperature occurs during an intermediate fraction (from *c* to *e*), the development is "behind schedule" at *c* (by the amount *c a* corresponding to the time *c d*), and on arrival at time *e*, calculated to give a certain fraction of the total developmental time, this will actually be fallen short of so far as concerns differentiation by the amount *h*, so that the degree of development is then in fact that represented at point d'. If now the organism be brought back to 20°, the developmental level is indicated at point *h*, which is ahead of that assumed at time *e;* during subsequent development at 20° the developmental course still to be completed is thus less than that calculated and hence appears faster; but with the conditions shown the apparent acceleration is less than in the first case, where transfer from the 10° to the 20° curve results in a considerable time-saving and therefore in a marked increase of calculated temperature coefficient.

(3) Again, if transfer from the 20° curve to the 10° be made late in development, as at point *m*, time is lost, because the relative develop-

ment is greater at the same time along the 10° curve; therefore the calculated temperature coefficient is actually less than that obtained from uninterrupted developments at 10° and at 20°.

For the prepupal period of *Drosophila*, with a normal temperature characteristic 16,850, Bliss (1925–26) found that in experiments of type (1) the apparent μ was 20,220; of type (2), 18,770; of type (3), 16,570. There is additional indication of just this sort of relationship in data given by Titschak (1926) for the rate of development in the clothes moth *Tineola*. The particular curves drawn in Fig. 3 are of course intended merely to show that it is possible to explain such results. It is significant that this explanation turns upon properties of an equation which describes with some precision the time course of growth and differentiation such as can be visibly evidenced by increasing weight, and which it is therefore legitimate to suppose may describe even more accurately the progress of developmental changes measurable only by the incidence of their end-results.

IV.

Although few investigations of growth permit very precise estimations of critical thermal increments, it is nevertheless important that there is indication of diverse magnitudes of μ, the temperature characteristic, for different cases; and that these values are suggestively close to those known to be associated with various other vital processes (Crozier, 1925–26). A careful investigation of a particular developmental stage in *Drosophila* has been made by Bliss (1925–26); Brown (1926–27) has determined the temperature characteristics for a developmental interval in cladocerans; the values of μ derived for these phenomena correspond to values repeatedly found in processes of quite different sorts (Crozier, 1925–26; Crozier and Stier, 1925–26). This is a striking fact, because one would be inclined to believe that chemical mechanisms controlling growth might be very different from those having to do, for example, with the regulation of the heartbeat or of breathing movements. This suspected difficulty might be overcome if it could be shown that the velocity of development, or the duration of life in particular stages, is determined, not by the magnitude of some simple underlying chemical change, but by the velocity with which this change is taking place. Northrop (1925–26) has indeed

shown that the duration of life in *Drosophila* does not depend upon the transformation of a definite amount of energy (Rubner), since the amount of CO_2 produced during life is not a measure of the life duration.

Aside from this possibility, however, the curve of equation (3) has some interesting properties which indicate another mode of interpretation. The reciprocal of the time for production of, say, $x = 0.90\,A$, will have a temperature coefficient which depends on the magnitudes of K_1, K_2, and A, and of the temperature coefficients of K_1 and K_2. If A be put = 100, and $K_1 = 0.010$, $K_2 = 0.005$, at 20°, with the respective temperature coefficients Q (10°–20°) = 2.0 and 2.94, then Q (10°–20°) for $1/t$ will be 2.74. Thus the temperature coefficient for $1/t$ may agree very closely with that for one of the two velocity constants.

This kind of relationship may be illustrated by one particular set of assumptions as to the values of A, K_1, and K_2, when it is supposed that $\mu = 11{,}300$ for K_1 and $\mu = 16{,}500$ for K_2. We desire to see whether the values of log $1/t$ will in such a case give a rectilinear relationship to the reciprocal of the absolute temperature, as seems to be the case in growth. It is seen that with very small deviation, such as would probably be overlooked in practical cases, they, indeed, do give this relationship (Fig. 4).[3] This is sufficient to demonstrate that an autocatalytic system in which two velocity constants are implicated may permit the approximate evaluation of temperature characteristics, and that these may even be quite close to those corresponding to the

[3] It is easily seen that with other values of K_1, K_2, the agreement could be very much closer. The values used in this illustration are chosen merely to show the slightly curvilinear character of the plot (Fig. 4), which would otherwise be detected with difficulty.

It may be pointed out that calculations of $1/$ *time for completion* of a stage of development based upon the integral form of $dx/dt = (K_1 + K_2\,x)\,(A - x)$ do not yield "breaks" in the curve of log $(1/$ *time*$)$ *vs.* $1/\,T°$ *abs.*, even when such temperature characteristics are assumed as reverse the sign of the difference between K_1 and K_2 at the extremes of temperature. In such a case the μ calculated from the times required to produce $x = 0.90\,A$ agrees rather closely with that μ, whether of K_1 or K_2, which happens to be smaller. It is of interest that in a system of this kind it is the magnitude of the *temperature characteristic*, rather than of the velocity constant, which chiefly determines the apparent μ of the resultant.

action of specific catalysts. The calculated curve is not exactly rectilinear, but in practice it may be very difficult to decide the origin of deviations at the extremes of the temperature range. It may be pointed out, however, that one may, from this standpoint, expect to find evidence of: (1) slight differences in critical increments when contrasting growth phenomena with other common activities, (2) relationships between log $1/time$ and $1/T°$ abs. which are not exactly rectilinear, but concave toward the $1/T°$ axis, and (3) deviations of critical increment when development is passed in part at one temperature, in part at another. The testing of these, especially (1) and (2), is at present handicapped by absence of precise data.

V.

In discussing growth rates as controlled by temperature it must be kept in mind that the momentary growth rate may vary with time. This may result not merely from the form of the growth-curve, but also because the growth-curve may smooth out recurrent cyclic variations in rate. An instance has already been given (Crozier, 1924–25, *b*), derived from Leitch's measurements of root elongation in the sweet pea. The temperature characteristics in this case differ markedly, depending upon the time period (0.5 hour or 24 hours) involved in the measurement. This is probably related to the fact that cell division occurs at a fairly definite point in a diurnal growth-rhythm (Stålfelt, 1921). Other kinds of complexity are not unexpected, for no one in his senses regards growth as an uncomplicated process. Lehenbauer's (1914) data show that the mean hourly elongation of maize seedlings increases abruptly at 20°, and with time. The average μ above 20° is about 15,500. Where such "breaks" occur in the temperature graph the effects to be expected when time is a significant variable are difficult to predict.

It is, nevertheless, of interest to examine the available measurements of growth to see if they yield anything in the nature of consistent temperature characteristics. A number of observers have studied the growth of fungal colonies as related to temperature. The data are not always presented in the most directly usable form, nor can the probable retarding effects of changes in the medium be discounted with any sureness. In the growth of such colonies on agar plates it is to be

supposed that in the absence of retarding or accelerating effects he rate of enlargement should be constant. A correct measure of growth, assuming only extension in area to occur, would be given by the increase in area per unit of circumference per unit time. When growth of such a colony gives a sigmoid curve with time it is certainly inap-

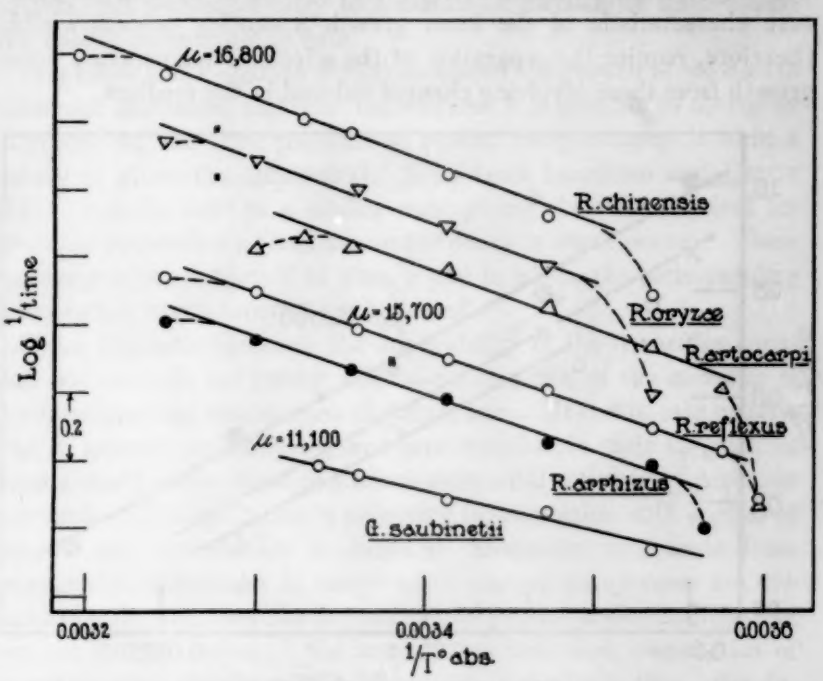

FIG. 5. The velocity of early growth in 5 species of *Rhizopus*, measured by the time required for the germinating hypha to become as long as the diameter of the spore (data from Weimer and Harter, 1923); and the growth of *Gibberella saubinetii*, taken as 1 ÷ time for colony to attain a diameter of 2.5 cm. (by interpolation from data of MacInnes and Fogelman, 1923). It is apparent that aside from terminal deviations such as are usually encountered the temperature characteristics closely resemble those calculated from data on respiration (Crozier, 1924–25, *b*).

propriate to regard the curve as describing an inner autocatalyzed growth-controlling process, and hence probably useless to attempt analysis of its precise relations to temperature. Fawcett (1921) records the growth in diameter of colonies of several fungi. These figures have been used to calculate the areal increase per unit time

(24 hours) per unit of growing edge at successive intervals. The rate of enlargement so computed changes with time, in such a way as to suggest that more extensive measurements would, at each temperature, pass through a maximum. The initial growth of the colony so affects the medium that growth is accelerated. Estimations of the temperature characteristic of the inner growth-promoting process would, therefore, require the separation of the effects of temperature upon growth from those involving changes induced in the medium.

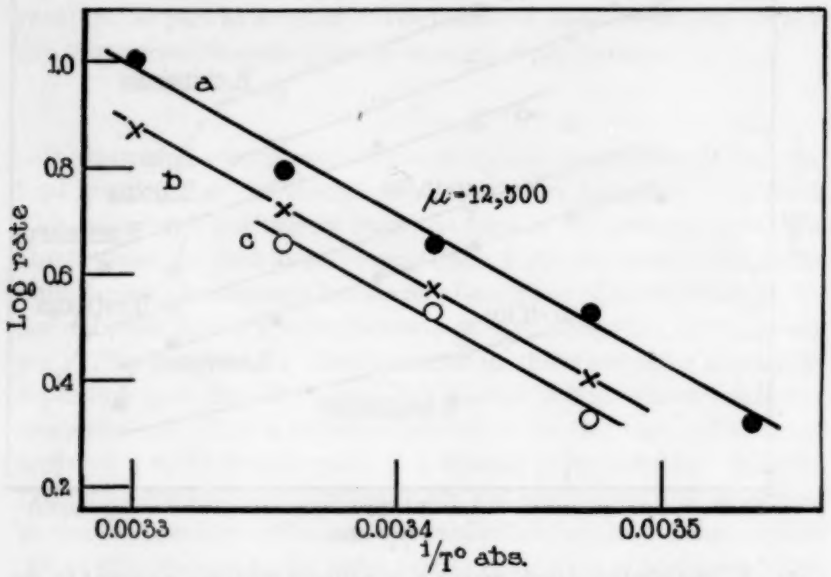

FIG. 6. The rate of decay of sweet potatoes inoculated with *Rhizopus*. The temperature characteristic is sensibly independent of the end-point chosen (*i.e.*, the amount of destruction, presumably determined in greater part by the activity of the fungus). Data from Lauritzen and Harter (1925).

Weimer and Harter (1923) studied the germination and growth of a number of species of *Rhizopus*. From their curves it is possible to plot the reciprocals of the times required for the germ tubes to grow until the length equals the diameter of the spores; these figures provide mean velocities of growth for the attainment of a constant amount of growth (Fig. 5). The agreement with the Arrhenius formula is usually excellent, since the deviations at high and at low temperatures

are no different from those generally encountered in other vital systems. The values of μ accord sufficiently well with those known in other growth phenomena.

In such a case it is to be presumed that the temperature characteristic obtained is not a property of the constants in an autocatalytic system, but pertains merely to a metabolic mechanism immediately responsible for increase in bulk.

MacInnes and Fogelman (1923) measured the growth of colonies of *Gibberella saubinetii*, and from their figures it is possible to obtain by interpolation the time required at several temperatures to form a colony of given size (diameter). Data from Lauritzen and Harter (1925) may be used in a similar way, giving the time required for *Rhizopus* to produce a given amount of decay in sweet potato. These measurements are plotted in Figs. 5 and 6, where the corresponding temperature characteristics are indicated.

These instances illustrate the applicability of the Arrhenius equation but they do not permit critical examination of the meaning of the corresponding temperature characteristics. It can be said merely, that in general the critical increments resemble in their magnitudes those already encountered in various other vital activities. A similar vagueness of interpretation is necessary in connection with studies of growth and regeneration in animals. Moreover, and aside from experimental difficulties in temperature control, many cases are disturbed by the fact that the developmental process under examination was not exactly begun at the temperature indicated, but the lot of organisms was distributed to thermostats some little time *after* development had begun. Bearing this difficulty in mind, we may, however, examine the velocity of development in several instances. It should be noted that there is direct justification for regarding a morphological end-point as a legitimate end-point for our purpose, provided it be timed with precision; Terroine and his associates (Terroine, Bonnet, and Joessel, 1924; Barthélemy and Bonnet, 1924) have indicated that thermal acceleration of development to a constant stage (*e.g.* to absorption of external gills, in the tadpole; germination of seeds) does not modify the utilization of energy during the growth process,—in other words, the chemical "balance" is the same at the same stage of development. But in many instances it may be impracticable to obtain a significant morphological end-point.

The speed of early cleavage of the fertilized ovum has been measured by Loeb and Wasteneys (1911) and by Loeb and Chamberlain (1915), for the egg of *Arbacia*. These two series of measurements are concordant, and exhibit critical increments 12,400; 21,000; 41,000,

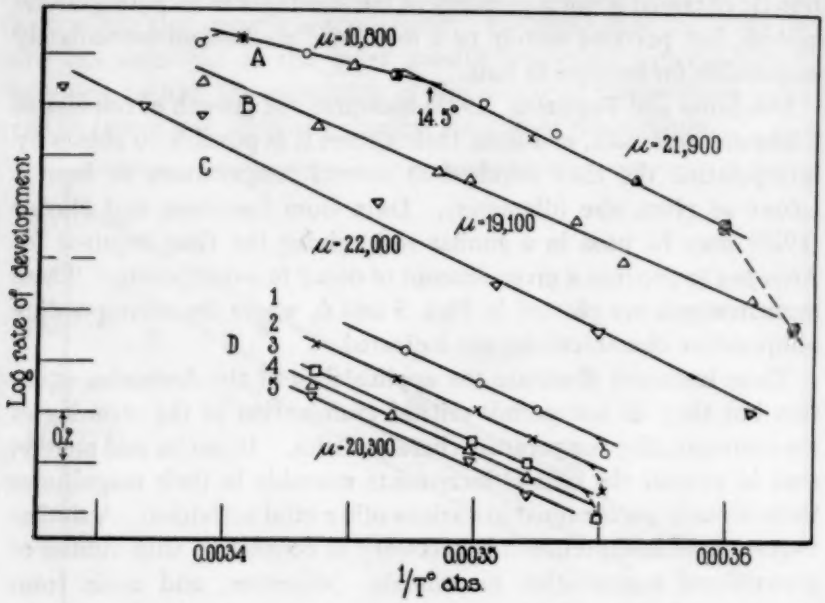

FIG. 7. Data upon the rate of cleavage and upon early stages of development in anurans.

A. Processes involved in the first cleavage, *Rana* (from Krogh, 1914).

B. From first cleavage to disappearance of yolk-plug (from Lillie and Knowlton, 1897).

C. From first, second, or third cleavage to disappearance of yolk-plug (Lillie and Knowlton, 1897).

D. From fertilization to: (1) medullary groove, (2) external gills, (3) 3 gill plumes, (4) a length of 7.0 mm., (5) a length of 7.8 mm.; *Rana* (Data from Krogh, 1914).

It is clear that there is a measure of consistency in the occurrence of $\mu = 20{,}000 \pm$. This is indicated also in some fragmentary data from Barthélemy and Bonnet (1924). Hertwig's data (*cf.* Cohen, 1901) also show parallelism in the effect of temperature at different stages of development, but indicate for the intermediate range of temperatures a distinctly lower μ, $17{,}000 \pm$. Estimations of the rate of growth of the body, and of the tail, in *Rana* and *Bufo*, agree in indicating $\mu = 24{,}000 \pm$ (data from Lillie and Knowlton, 1897).

with "breaks" at 11° and at 20° (Crozier, 1924–25, *b*). The velocity of segmentation in eggs of the frog (first cleavage) is plotted in Fig. 7, from data by Krogh (1914). The velocity of subsequent early

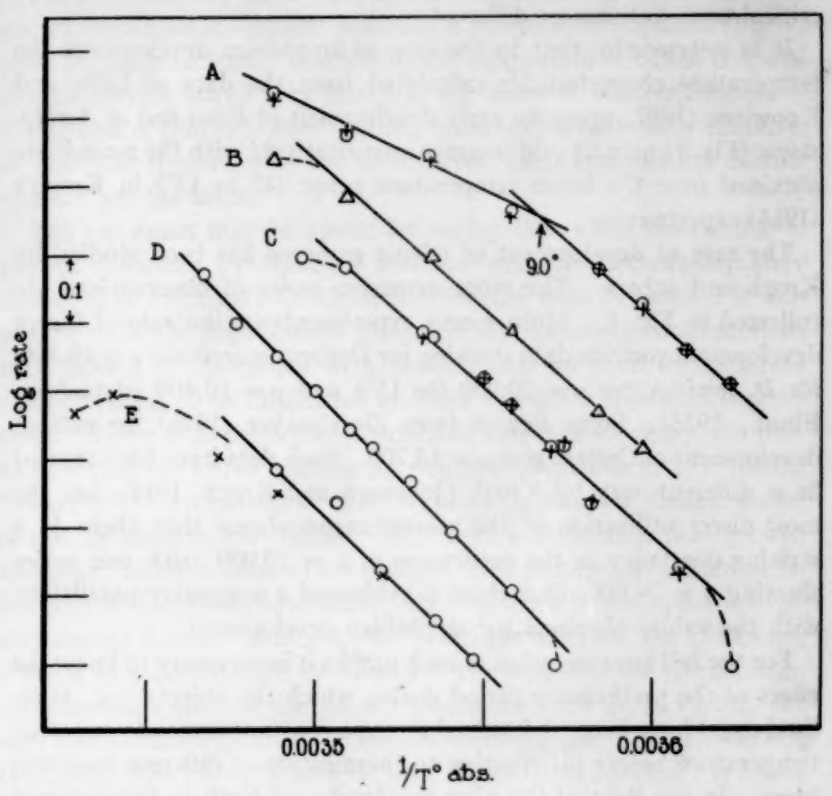

FIG. 8. Data upon the development of teleosts:

A, *Gadus morrhua* and *G. æglefinus*; *B*, *G. merlangus*; average velocities of development up to hatching; observations by Dannevig (in Johansen and Krogh, 1914); for *A*, μ = 11,800 and μ = 20,200, with break at 9.0°C; for *B*, μ = 20,000.

C, Plaice; to length 4.6 mm., circles; to length 4.9 mm., crosses; the two series brought together by multiplying the members of the second by a factor; μ = 20,000. (Johansen and Krogh, 1914).

D, *Hypomesus olidus* (Higurashi and Tauti, 1925); μ = 23,700.

E, *Plecoglossus altivelis*; two series, one from Higurashi and Tauti (1925), the second from Higurashi and Nakai (1926); μ = 23,000.

It should be noted that in the cases of deviation at extreme temperatures there is independent evidence of abnormal differentiation.

development of the frog, in Krogh's experiments, exhibits a constant increment not significantly different from that for the mid-range of temperatures (15° to 4.7°) in the case of the first cleavage, but the critical temperatures are different.

It is noteworthy that in the case of amphibian development the temperature characteristics calculated from the data of Lillie and Knowlton (1897) upon the early development of *Rana* and of *Amblystoma* (Fig. 7) may be said to agree quantitatively with the magnitude obtained over the lower temperature range (4° to 15°) in Krogh's (1914) experiments.

The rate of development of teleost embryos has been studied by Krogh and others. The more extensive series of observations are collected in Fig. 8. More recent experiments on the rate of insect development provide data showing for *Dytiscus marginalis* $\mu = 19{,}300$, for *D. semisulcatus* $\mu = 20{,}000$ (to 15°) and $\mu = 10{,}400$ (data from Blunk, 1923). Some figures from Ziegelmayer (1926) for rate of development of *Cyclops* give $\mu = 15{,}700$. Such data have been treated in a different way by Krogh (Johansen and Krogh, 1914), but the most direct utilization of the measurements shows that there is a striking constancy in the occurrence of $\mu = 20{,}000$, with one series showing $\mu = 24{,}000$; thus there is evidenced a suggestive parallelism with the values obtained for amphibian development.

For the full interpretation of such graphs it is necessary to know the effect of the preliminary period during which the objects (*e.g.*, fertilized eggs) have been maintained at some constant or nearly constant temperature before distribution to thermostats at different temperatures. In the light of the view previously set forth in this paper it would be expected that if at all prolonged this preliminary interval might well have a definite and detectable effect. It would be expected to accentuate any innate departure from the rectilinear character of the plots, or might seriously affect the apparent magnitude of the temperature characteristic. This especially makes it impossible to use critically much of the early material on insect development (*cf.* Sanderson, 1910; Sanderson and Peairs, 1913), although it supplies interesting suggestions. In the most carefully conducted experiments the

adherence to rectilinearity is certainly satisfactory (*cf.*Crozier, 1924–25, *b*; Bliss, 1925–26; Brown, 1926–27).

The fact that the same temperature characteristic holds for mean growth velocities at different points on a curve of development (Fig. 7) must be taken to signify that within the range considered the shape of the underlying curve very nearly is the same at different temperatures; hence, that only one "velocity constant" is materially effective, or else that if more than one be involved, their temperature characteristics are the same.[4]

The net result may be stated by saying that while the control of growth velocities by chemical reaction velocities seems adequately shown, it is yet highly desirable that further data be secured by improved methods; there is some indication that the controlling reactions may belong in categories with those found by their temperature characteristics to be implicated in other and quite diverse vital processes. There is as yet scarcely sufficient evidence to verify the prediction that the curve relating log velocity of growth to $1/T°$, when "velocity" = reciprocal of time required to reach a defined stage, should be slightly curvilinear. But there is indication that growth velocities, where evidenced as constant rates of increase, adhere satisfactorily to the Arrhenius formula; and even when we may quite reasonably expect that an "autocatalytic" system is involved, the agreement is often as good as might be desired. The values of the temperature characteristics secured for growth phenomena are quite varied, yet they cluster rather definitely about the following magnitudes; 7–8,000; 11–12,000; 16–17,000; 20,000; 24,000; 27,000 (this summary is based upon studies of a number of cases additional to those specifically mentioned in this article). Consideration of the properties of the equation which describes the velocity of an autocatalytic process (Section IV) shows how this sort of result may be obtainable.

[4] With regard to velocities of regenerative growth, which in certain cases at least appear to adhere to typical growth curves, there does not exist any considerable body of data. We may cite the following instances. The regeneration of hydranth in *Tubularia* (Moore, 1910) has been cited in an earlier paper (Crozier, 1924–25, *b*). Measurements of the rate of regeneration (morphyllaxis) in planarians indicate a high temperature coefficient (Lillie and Knowlton, 1897; Vandel, 1921–22), but are insufficient for analysis.

VI.

SUMMARY.

The velocity of growth, taken as the reciprocal of the time required to attain a given size or stage of development, obeys with some exactness the Arrhenius equation for relation to temperature. The values of μ, and the type of "breaks" found in the curves connecting velocity and temperature, are similar to those found in the case of various other vital activities. More precise data, particularly from experiments in which parts of the given developmental stadium are passed at different temperatures, may strengthen present indications that this relationship is not absolute. It is pointed out that the equation for an autocatalytic process, taken as descriptive for growth, predicts particular sorts of deviation under these conditions, which have in one instance been obtained experimentally; and may at the same time nevertheless permit the apparent temperature characteristic for (average) growth velocity to agree rather closely with that for one of the two velocity constants present in the correct autocatalytic equation.

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EXIT OF DYE FROM LIVING CELLS OF NITELLA AT DIFFERENT pH VALUES.*

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(Accepted for publication, July 6, 1926.)

I.

INTRODUCTION.

The purpose of the present paper is to outline a theory[1] of the penetration of a dye (brilliant cresyl blue) into living cells of *Nitella*,[2] and to examine how far this theory is in harmony with the facts found in studying the exit of the dye from the cell.

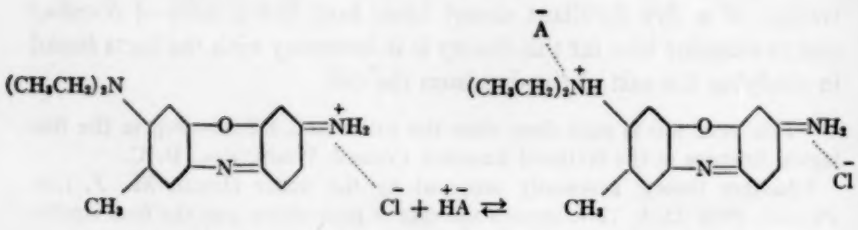
* This work was in part done when the writer held a Fellowship in the Biological Sciences of the National Research Council, Washington, D. C.

[1] Another theory, previously proposed by the writer (Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 727), regards the rate of penetration and the final equilibrium as dependent primarily on the concentration of the salts of proteins or weak acids, XA, at the surface of the cells, which combine with DS, to form a compound capable of diffusing into the sap. But on further experimentation the writer has concluded that there are many objections to this theory. The most serious objection of all is found in the fact that the rate of penetration seems to be directly proportional to the ratio $\frac{DB}{DS}$ for each particular dye at various pH values, when a comparison of the relative rates at these pH values is made by the writer among several basic dyes having different apparent dissociation constants.

Penetration is regarded by the following writers as dependent on the combining of dye ions with proteins: Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354. Mathews, A., *Am. J. Physiol.*, 1898, i, 445.

[2] *Nitella* is a fresh water plant with multinucleate cells up to 4 inches in length, having an outer cell wall, beneath which is a very thin layer of protoplasm surrounding a relatively large central vacuole. The pH value of the sap in the vacuole is about 5.6, and the sap contains about 0.1 M halides in addition to organic acids and protein.

The theory [3,4] states that the dye exists in (at least) two forms, one of which is the "free base"[5] which we may call DB, soluble in chloroform, and another, which we may call DS, little or not at all soluble in chloroform. When the pH value of the solution increases a part of DS is changed to DB (and *vice versa*), so that at each pH value these two forms are in equilibrium with each other (and possibly with a third form, which may be a pseudo base found at still higher pH values). The nature of DB is still an open question. According to the theory of Hantzsch and others[6] both DB and DS may be treated as salts, DB being a quaternary ammonium salt which is capable of undergoing a further salt formation owing to the presence of other basic groups. The following[7] may make this clear by using cresyl blue as an example.



DB: colored blue in water and red in chloroform. Soluble in chloroform and water.

decreasing pH →

← increasing pH

DS: colored blue in water. Soluble in water but little or not at all in chloroform.

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[3] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

[4] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561.

[5] In former papers (see Foot-notes 3 and 4) this free base was called DOH for convenience but in order to avoid any possibility of confusing DOH with the dye hydrate (which may not be the form we are dealing with), it will be called DB hereafter.

[6] For a discussion of the theory of indicators see Henrich, F., Theories of organic chemistry, translated by John Johnston and Dorothy Hahn, London, 1922.

[7] For the formula see Conn, H. G., Biological stains, Geneva, New York, 1925, 51.

In the case of brilliant cresyl blue, DB and DS have the same color. If the above description of DB and DS is correct DB may be a strongly dissociated salt like DS. On the other hand, DB may be regarded as an undissociated molecule, and DS a strongly dissociated salt. Experiments are being carried out by the writer to determine the behavior of DB in this respect. Dr. Grinnell Jones has kindly determined the change in the conductivity of chloroform with and without the dye. When 100 cc. of pure chloroform were shaken up with 1 liter of M/150 borate buffer solution at pH 9, the specific conductivity of this chloroform was found to be 6×10^{-10}. When the same volume of chloroform was shaken up with 1 liter of M/150 borate buffer solution at pH 9 containing 3.5×10^{-4} M brilliant cresyl blue until there was practically no dye left in the aqueous solution, the specific conductivity of this chloroform was found to be 233×10^{-10} (about forty times greater than that of the chloroform containing no dye). This indicates that some or all of the dye exists in the chloroform in dissociated form.

The behavior of these two forms is very different. Apparently[8] DB can pass through the cell rapidly but DS penetrates extremely slowly or not at all.

Although it is evident that the form of the dye[9] which principally

[8] In connection with this, it may be assumed that DS corresponds with the ions and DB with the undissociated molecules, of a weak base, acid, or salt. In the paper by Hoagland and Davis (Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, vi, 47) it is stated that the time of exposure of the living cells of *Nitella* to solutions containing NO_3 or Br ions, is a matter of days, before a detectable amount is found in the sap even at a favorable external pH value, temperature, and condition of light. The time of exposure, on the other hand, in the case of the penetration of cresyl blue into *Nitella* at a favorable external pH value, and temperature, is a matter of seconds. This fact agrees very well with Osterhout's suggestion (see Foot-note 10) that the undissociated molecules enter the cell, while the ions enter only very slowly or not at all. Furthermore, it agrees with the writer's theory, since the halides are only very slightly soluble in substances like chloroform and benzene, and in this respect the halides correspond with DS of the basic dye.

[9] The following writers state that some basic dyes enter the living cells as a free base: Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501. It is stated by Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360, that an acid dye, 2,6,dibromophenol indophenol penetrates *Valonia* only in the form of an undissociated molecule.

penetrates the cell is the one which is soluble in chloroform, the writer does not wish to subscribe without reserve to the lipoid theory in its present form.

Previous experiments[1,2] have shown that when the external concentration is kept constant throughout the experiment, the entrance of the dye is found to follow the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

where a = the concentration of the dye in the sap at equilibrium and x = the concentration of the dye in the sap at the time t, while k = the velocity constant. When the values of x are calculated from this equation they are found to agree very closely with the observed.

This agreement of course does not determine whether the process is governed by diffusion or by chemical reaction.

The temperature coefficient, furthermore, for the rate of penetration between 20°C. and 25°C. is very high (above 4) but this again may not necessarily indicate that the process is controlled by a chemical reaction rather than by diffusion.

Until further knowledge is obtained concerning the temperature coefficient for the diffusion of substances through an artificial system which more or less closely resembles the living cell of *Nitella*, and in which the passage of solute molecules or ions from one solvent phase to another probably does not depend upon forces of the sort usually regarded as "physical" it is not possible to determine whether the rate is governed by simple diffusion or by chemical reaction.

It is quite possible that under some circumstances it is controlled by diffusion and under other circumstances by chemical combination.

Since we are unable at present to decide whether the rate is controlled by diffusion or by chemical reaction let us for the sake of simplicity assume that it is diffusion, since in this case the mechanism is less complicated, and proceed to analyze the data on this basis. After this is done we shall discuss the alternative hypothesis; *i.e.*, that the rate is controlled by chemical reaction.

If we assume that the rate is controlled by diffusion, the mechanism may be explained as indicated by Diagram A (the cell wall being omitted). In this diagram nothing is said regarding combination of

the dye with a cell constituent but this does not indicate that there is no possibility of such a reaction in the protoplasm. It is regarded, for the present, as not affecting the rate; it is therefore omitted in order to simplify the diagram.

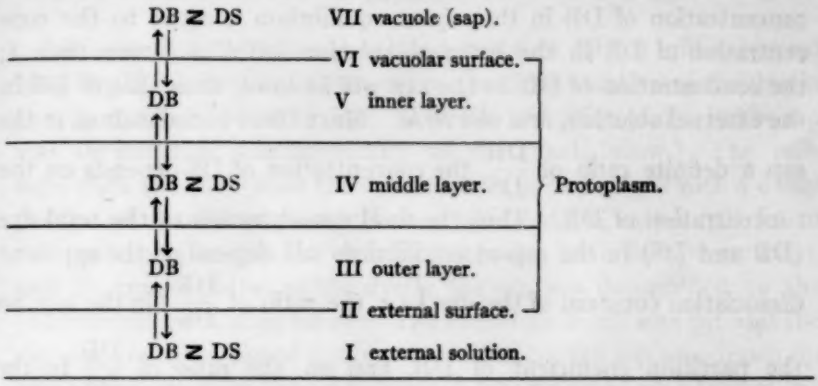

Diagram A. The cell wall is omitted for convenience.

Diffusion is designated by the sign ⇄ ; equilibrium between DB and DS by the sign ⇄. For convenience the process is divided into seven parts. The inner and outer layers (III and V) are hypothetical. The vacuolar surface (VI) represents the protoplasmic surface in immediate contact with the sap, while the external surface (II) represents the protoplasmic surface in contact with the external solution. In I, IV, and VII, DB is in equilibrium with DS and a constant ratio of $\frac{DB}{DS}$ is maintained in each medium as long as the conditions remain unchanged. The concentration of DB in one part is in definite relation with that of DB in any other part of the diagram. Thus, for example, if the concentration of DB in the external solution (I) is changed, successive changes in the concentration of DB in all the parts of the cell take place. It is assumed that DB diffuses through III and V while DS diffuses to such a slight extent as to be negligible in the present case. For penetration the velocity of diffusion of the dye, DB, from I to VII is greater than that from VII to I while for the exit the velocity from VII to I is greater. An equilibrium is established when the velocity of the inward diffusion is equal to the outward

diffusion. At equilibrium the concentration of DB in the vacuole is proportional to the concentration of DB in the external solution depending on the apparent[10] dissociation constant and on the distribution coefficient $C = \frac{\text{DB in the sap}}{\text{DB in the external solution}}$. If C is 1, the concentration of DB in the sap at equilibrium is equal to the concentration of DB in the external solution. If C is lower than 1, the concentration of DB in the sap will be lower than that of DB in the external solution, and *vice versa*. Since there is maintained in the sap a definite ratio of $\frac{DB}{DS}$, the concentration of DS depends on the concentration of DB. Thus the final concentration of the total dye (DB and DS) in the sap at equilibrium will depend on the apparent dissociation constant of the dye $\left(i.e.\text{ the ratio of } \frac{DB}{DS}\right)$ in the sap, on the partition coefficient of DB, and on the ratio of $\frac{DB}{DS}$ in the external solution.

According to this scheme it is possible to study the mechanism either of the penetration of the dye into or of the exit from the vacuole, by determining the concentrations of the dye (DB plus DS) in the sap, as long as the color of DB does not differ from that of DS. The rate of penetration will increase and that of exit will decrease when the concentration of DB just outside the external surface (II) is increased. The reverse is the case when the concentration of DB in the sap is increased as, for example, by any change in the medium which changes the apparent dissociation constant of the dye $\left(i.e.\text{ the ratio of } \frac{DB}{DS}\right)$, or by a change in the solubility of DB in the sap.

The theory thus outlined accords with the facts previously obtained for the penetration of dye.[3,4] Let us now consider whether it accords with the facts observed in connection with the exit of dye from the cell. We shall proceed upon the assumption that when a stained cell is placed in a solution containing no dye, the dye comes out according to

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

the scheme outlined in Diagram A (the outward process VII I from the sap to the external solution). We shall test this assumption by experiments.

II.

Methods.

Living cells of *Nitella* were placed in 8.6×10^{-6} M brilliant cresyl blue at pH 8.2 for 17 minutes, when the concentration of the dye in the sap reached 7.94×10^{-5} M. (The concentration of dye in the sap was determined colorimetrically as described below.) The cells were then removed from the dye solution, gently wiped with a damp cloth, and distributed in solutions at different pH values (pH 5.4 to 8.2) containing no dye. At definite intervals a few cells were removed, and the concentration of the dye in the sap was determined by the colorimetric method as follows: The end of each cell was cut and the sap was gently squeezed out onto a glass slide; the sap was drawn up into a capillary tube the color of which was matched with that of the capillary tube of the same diameter containing a standard dye solution.

In order to avoid experimental error from the presence of the dye in the external solution, only six cells were placed in 200 cc. of solution (without dye) and the solution was constantly stirred and changed every 15 seconds. This method gives the maximum velocity constant for each experiment, *i.e.* there is no further increase in the velocity of the exit of the dye if the frequency of stirring and of changing the solutions is increased.

The concentration of the external dye solution (8.6×10^{-6} M) is chosen because this is sufficiently dilute to avoid error due to the adhering of the dye to the surface of the cell, after the cell is removed and wiped with a damp cloth. The cellulose wall is not stained when cells are placed in this concentration of dye at pH 8.2.

If too high an external dye concentration is used, the exit of the dye from the sap is hindered (even when the cell wall is not stained) when the cells are removed from the dye solution and placed in a solution without dye, though the latter solution is constantly stirred and changed. This decrease in the rate of the exit of the dye is due in all probability to the fact that the dye adhering to the surface of the cell cannot be washed away quickly enough. This complication may

be eliminated by using an external solution which is at least seven times more dilute than the concentration of the dye in the sap which is chosen for the experiments. In order to be absolutely certain that the concentration used avoids this experimental error the experiment was repeated with still lower concentrations but it was found that the result was not altered.

All possible care was taken to have all the cells used at one time as alike as possible, so that the differences in the rates were due chiefly to the experimental conditions and not due to the difference in the condition of the cells before the experiments began. Unless otherwise stated, the *Nitella* used was obtained from Cambridge and the experiments were carried out in early fall when the cells were in excellent condition.

The tests for early stages of injury are very unsatisfactory. The appearance of masses of chlorophyll in the expressed sap, the rapid exit of halides from the intact cell, and the loss of turgidity all indicate advanced stages of injury rather than the first. For this reason it is desirable to control the experiments in some way so that we have a more or less uniform method of detecting the condition of the cell immediately after the experiments. To do this, after each experiment, some of the cells were tested for injury by placing them in distilled water, and for 4 days[11] at intervals of every few hours the percentage mortality was compared with that of the control cells (fresh cells placed in distilled water under same conditions). It was found that the percentage mortality of the cells thus treated was about the same as that of the control cells.

These experiments, like those heretofore described[3,4] by the writer, were carried out in an incubator at 25 ± 0.5°C., into which diffused light was permitted to enter through small ventilating holes.

The buffer solutions used were M/150 phosphate mixtures. The pH values of these buffer solutions were determined by means of the hydrogen electrode. The dye used was that of Grübler, and was taken from the same stock bottle as the one used in the writer's experiments[3,4] on penetration.

[11] It is not desirable to continue such a test for any longer period since the comparison between the test cells and the control cells becomes more doubtful, in view of the fact that even the control cells do not live indefinitely in the laboratory.

III.

Analysis of the Time Curves.

That lowering of the pH value of the external solution (containing no dye) hastens the exit of the dye from the sap of living cells of *Nitella* is indicated[12] by the curve in Fig. 1. At low pH values (5.4 to 6) the process may be followed until practically all the dye has come out of the sap without causing injury to the cells, but at higher pH values injury or death may occur. The curves given in Fig. 1 represent the process when the cells are not injured.

At higher pH values of the external solutions here employed it is probable that all the dye in the sap would eventually be found to come out of the vacuole if we could continue the experiment long enough and still keep the cell from being injured. The analysis of the time curves therefore is made on the assumption that at the end of the process the concentration of the dye in the sap is zero at all external pH values.

The velocity of diffusion is assumed to be proportional to the difference between the concentration of DB in the sap and that of DB in the external solution. According to the present theory, there is a definite ratio of $\frac{DB}{DS}$ in the sap and in the external solution, so that for mathematical treatment the concentration of DB in both may be replaced, for convenience, by the concentration of the total dye (DB and DS) which we actually measure. Since the concentration of the dye in the external solution is approximately zero, we may in the following equation let a denote the initial concentration of the dye in the sap, x the concentration of the dye that has disappeared from the sap at time t, and k the velocity constant of diffusion. We may then write:

$$\frac{dx}{dt} = k\,(a - x) \quad \text{or} \quad k = \frac{1}{t} \log \frac{a}{a - x}$$

When k is calculated for each time curve it is found to decrease

[12] These results confirm those obtained previously by the writer (Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223). It may be added here that the writer has chosen to study the exit of the dye by the method presented in this paper first, because other methods offer greater complications.

TABLE I.

Exit of Brilliant Cresyl Blue from Living Cells of Nitella at Varying External pH Values at 25°C.

The process is represented by the equation $\frac{dx}{dt} = k(a - x)$, where a is the initial concentration of dye in the sap, $a - x$ is the concentration of dye in the sap at the time t, and k is the velocity constant. The calculation is made with a 20 inch slide rule. $a = 7.94 \times 10^{-5}$ M for all external pH values.

t	pH 5.4			pH 5.7			pH 6.0			pH 6.8			pH 7.5			pH 7.8			pH 8.2		
	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.	$a-x$ obs.	k	$a-x$ calc.
min.	M $\times 10^5$		M $\times 10^5$	M $\times 10^5$		M $\times 10^5$	M $\times 10^5$		M $\times 10^5$	M $\times 10^5$		M $\times 10^5$	M $\times 10^5$		M $\times 10^5$	M $\times 10^5$		M $\times 10^5$	M $\times 10^5$		M $\times 10^5$
1	4.83	0.215	4.96	4.83	0.215	5.07	5.59	0.152	5.62	6.21	0.107	6.30									
2	3.05	0.208	3.11	3.28	0.192	3.23	4.00	0.148	3.96	4.83	0.107	5.01	6.14	0.055	6.02	6.56	0.041	6.46	6.9	0.030	7.08
3	2.15	0.189	1.94	2.31	0.179	2.06	2.76	0.153	2.81	4.14	0.094	3.98	5.00	0.067	5.24	5.87	0.043	5.82	6.80	0.023	6.68
5							1.40	0.151	1.40	2.76	0.092	2.51	3.80	0.064	3.97						
7													3.28	0.055	3.02	3.80	0.046	3.84	5.52	0.023	5.31
10																2.59	0.049	2.82	4.49	0.025	4.47
Average.....		0.204			0.195			0.151			0.100			0.060			0.045			0.025	

slightly as the concentration of the dye (DB and DS) in the sap approaches zero (Table I). This decrease in the value of k may be explained on the ground that there is a relative increase in the velocity of the inward process due to an increase in the ratio of DB in the film just outside the external surface (II) to the DB in the outer layer (III) and hence to the DB or to the total dye (DB and DS) in

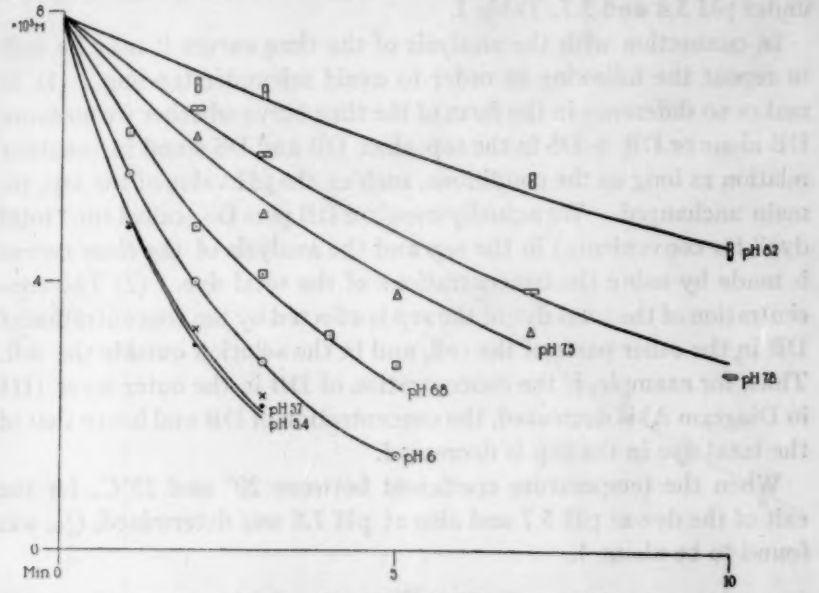

FIG. 1. Time curves showing the exit of brilliant cresyl blue from the living cells of *Nitella* at different external pH values at 25°C., when the initial concentration of the dye in the sap is 7.94×10^{-5} M. The ordinates represent the concentrations of dye in the sap, while the abscissæ represent time. The curves as drawn represent the calculated values of the concentration of the dye in the sap, while the symbols represent the observed values. Each point on every curve is an average of fifty experiments, and the probable error of the mean is less than 8 per cent of the mean.

the sap, since toward the end of the process, where there is a very little dye (DB and DS) left in the sap, the amount of DB in the film just outside the external surface (II) may no longer be a constant fraction of DB in the outer layer (III) and of the total dye in the sap as was the case at the beginning of the process (this will be discussed later on). Since this decrease in the con-

stants is not very great, the average is taken of all the velocity constants at each external pH value. When the values of $a - x$ are calculated for each time curve by using the average value of k, thus obtained, they are found to agree fairly closely with the observed except toward the end of the process, where there is an indication that the calculated values are slightly lower than the observed, as shown under pH 5.4 and 5.7, Table I.

In connection with the analysis of the time curves it may be well to repeat the following in order to avoid misunderstanding. (1) It makes no difference in the form of the time curve whether we measure DB alone or DB + DS in the sap, since DB and DS stand in constant relation as long as the conditions, such as the pH value of the sap, remain unchanged. We actually measure DB plus DS (called the "total dye" for convenience) in the sap and the analysis of the time curves is made by using the concentrations of the total dye. (2) The concentration of the total dye in the sap is affected by the concentration of DB in the other parts of the cell, and in the solution outside the cell. Thus, for example, if the concentration of DB in the outer layer (III in Diagram A) is decreased, the concentration of DB and hence that of the total dye in the sap is decreased.

When the temperature coefficient between 20° and 25°C., for the exit of the dye at pH 5.7 and also at pH 7.8 was determined, Q_{10} was found to be about 4.

IV.

The Relation of the Velocity Constant to the pH Value of the External Solution.

The time curve for each external pH value is found (see Table I) to follow approximately the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

where a denotes the initial concentration of DB in the vacuole minus the concentration of DB in the external solution (which in this case is practically zero), x the amount of DB that has diffused out of the vacuole at the time t, and k the velocity constant. In both cases, DB for convenience is put equal to the total dye which is actually measured.

If the stirring and frequent changing of the external solution kept the concentration of DB equal to zero just outside the external surface of the protoplasm (which is designated by II, the external surface, in Diagram A), we should expect to find the same values of k for all external pH values. But the value of k decreases with an increase in the external pH value, as shown in Fig. 2, and the explanation for

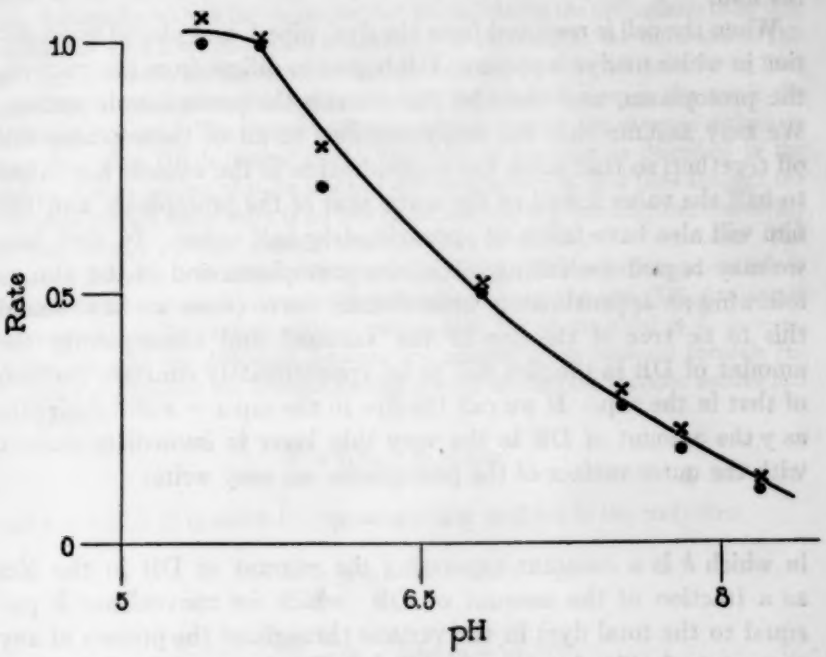

FIG. 2. Curve showing the relation of the external pH values to the rates of the exit of the dye (symbol ×), and also to the velocity constants (symbol ●). The ordinates represent the rates, and also the velocity constants multiplied by 5.2 (for convenience of plotting), and the abscissæ represent the pH values of the external solutions.

this may be that the concentration of DB at the external surface changes as the external pH value changes. Let us assume that just outside the external surface of the protoplasm there is a liquid film more or less protected against the direct effect of stirring by the cellulose wall and that in this film a certain amount of DB (a certain percentage of which is at once transformed to DS) collects as it comes out of the cell; also that the total amount of DB

which comes out is approximately the same in all solutions containing no dye, but the per cent of it which remains in the form of DB depends on the pH value of this film (which is assumed to be approximately the same as that of the external solution since the latter can penetrate freely through the cellulose wall into the film) since a certain amount of DB will change to DS depending on the pH value of the film.

When the cell is removed from the dye, wiped, and placed in a solution in which no dye is present, DB begins to diffuse from the vacuole, the protoplasm, and the film just outside the protoplasmic surface. We may assume that the concentrations in all of these places fall off together, so that when the concentration in the vacuole has fallen to half the value it had at the start, that of the protoplasm and the film will also have fallen to approximately half value. In that case we may regard the falling off in the protoplasm and in the film as following an approximately unimolecular curve (since we have found this to be true of the dye in the vacuole) and consequently the amount of DB in the film will be an approximately constant fraction of that in the sap. If we call the dye in the sap $a - x$ and designate as y the amount of DB in the very thin layer in immediate contact with the outer surface of the protoplasm we may write:

$$y = b\,(a - x)$$

in which b is a constant expressing the amount of DB in the film as a fraction of the amount of DB (which for convenience is put equal to the total dye) in the vacuole throughout the process at any one external pH value.

When the pH value of the external solution changes the value of b will also change, since the per cent of DB in the film will be altered. In order to see how this will affect the rate of exit of the dye from the vacuole, let us first consider the case where there is no effect of y on the velocity constant. Since according to our analysis of the time curves, the dye comes out of the vacuole in a unimolecular fashion, we may write

$$\frac{dx}{dt} = k_1\,(a - x)$$

in which k_1 is the velocity constant of the process when dye is present

on one side of the surface only. This expression gives us the rate of exit of the dye when there is no dye in the film. When dye is present in the film a certain amount diffuses back into the cell. The true rate[13]

[13] Criticism may be made as to this method of mathematical treatment since it involves the consideration of the diffusion of DB through only one very thin surface, when in fact the protoplasm of *Nitella* consists of more than one such layer. Even if we were to treat the entire protoplasmic layer as one surface, the question may be raised as to how far we are justified in considering the protoplasm to be thin enough for such a mathematical treatment. If we consider the diffusion of DB through two surfaces, the vacuolar and the external surfaces (II and VI in Diagram A), one at a time, then we may modify the analysis given in the text in the following manner. The amount diffusing inward through the external surface in unit time when DB is present in the film only $= k_1 y$ (just as described in the text). Let us assume that the amount diffusing inward in unit time through the vacuolar surface when there is no DB in the vacuole is a constant fraction of $k_1 y$ so that we may put this amount equal to $ck_1 y$, in which c is a constant. The amount diffusing outward in unit time through the vacuolar surface when DB is present in the vacuole but not in the protoplasm or in the film (DB fictitiously introduced into the vacuole without getting into the protoplasm) is $k_1 (a - x)$. Hence we take the difference between the amount going outward through the vacuolar surface and the amount passing inward through the vacuolar surface and we have:

$$\frac{dx}{dt} = k_1 (a - x) - k_1 cy$$

put $y = b (a - x)$ in which b is a constant (just as given in the text) then

$$\frac{dx}{dt} = k_1 (a - x) - k_1 bc (a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1 bc) (a - x)$$

or on integration

$$k_1 - k_1 bc = \frac{1}{t} \log \frac{a}{a - x}$$

$$bc = \left(k_1 - \frac{1}{t} \log \frac{a}{a - x} \right) \div k_1$$

Since we are not able to verify the values of the constants b and c experimentally, and since assuming a value for either b or c is very unsatisfactory, we are not able to explain the mechanism any more convincingly than we have done in the text.

Experiments are now in progress to see whether it is possible to determine the

of exit is the resultant of these two processes and may be found by subtracting the amount which would diffuse inward if dye were present on one side only from the amount that would diffuse outward if dye were present on the other side only. Hence we may write

$$\frac{dx}{dt} = k_1 (a - x) - k_1 y$$

Substituting in this equation the value $y = b (a - x)$ we have

$$\frac{dx}{dt} = k_1 (a - x) - k_1 b (a - x)$$

$$\frac{dx}{dt} = (k_1 - k_1 b) (a - x)$$

or, on integration,

$$k_1 - k_1 b = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 (1 - b) = \frac{1}{t} \log \frac{a}{a - x}$$

$$k_1 = \left(\frac{1}{t} \log \frac{a}{a - x}\right) \div (1 - b)$$

and

$$b = \left(k_1 - \frac{1}{t} \log \frac{a}{a - x}\right) \div k_1$$

We may put $k_1 (1 - b) = k$; substituting the value $k = \frac{1}{t} \log \frac{a}{a - x}$ we have $b = \frac{k_1 - k}{k_1}$.

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constants experimentally in order that we may know in greater detail what the controlling factor is for the rate of penetration into and that of exit of the dye from the vacuole.

It might be possible that the rate of penetration into and that of the exit of the dye from the vacuole are controlled by the rate of diffusion of DB through only one very thin layer in the cell, (the layer through which the diffusion of DB is the slowest). Whether this is represented by the external surface (II in Diagram A) or by the vacuolar surface (VI) or by some other part of the cell, we are not able to state definitely at present. In all probability under varying conditions the controlling layer varies.

The values of a, x, and t may be obtained experimentally but the values of k_1 and b cannot be obtained in this way. We may, however, assume an approximate value of k_1 and we are justified in doing this since we are interested in relative rather than in absolute values. The analysis of the time curves shows decreasing values of k (see Table I and Section III) with increasing external pH values. When such

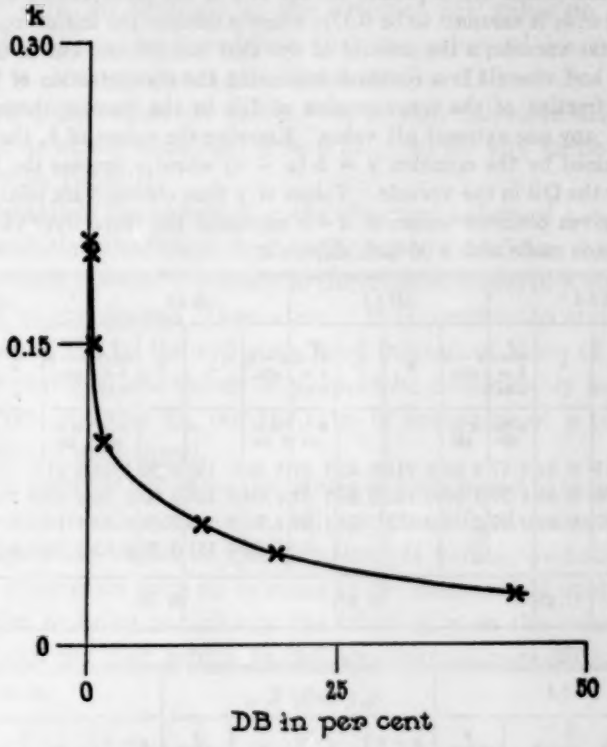

FIG. 3. Curve showing the relation of the velocity constants (k), plotted as ordinates, to the concentrations of free base (DB), in per cent as abscissæ.

values of k are plotted as ordinates and the concentration of DB in the external solution expressed as per cent of the total dye, (obtained from the experiments[4] made by the writer on the distribution of the dye between chloroform and water) are plotted as abscissæ, we obtain the curve shown in Fig. 3. The curve indicates that when DB (and consequently y) equals zero, the value of k is somewhat above

TABLE II.

The Relation of the Dye in the Film Just Outside the Protoplasmic Surface to the Dye in the Vacuole, at Different External pH Values.

The values of b are obtained by the equation $b = (k_1 - \frac{1}{t} \log \frac{a}{a-x}) \div k_1$ where k_1 denotes the velocity constant of the diffusion of the dye into or out of the living cell of *Nitella* where dye is present on one side of the protoplasmic surface only (the value of k_1 is assumed to be 0.37); where a denotes the initial concentration of dye in the vacuole; x the amount of dye that has diffused out of the vacuole at time t; and where b is a constant expressing the concentration of DB in the film as a fraction of the concentration of DB in the vacuole throughout the process at any one external pH value. Knowing the values of b, the values of y are obtained by the equation $y = b\ (a - x)$ where y denotes the DB in the film, $a - x$ the DB in the vacuole. Values of y thus obtained are relative values, since the given observed values of $a - x$ represent the "total dye" (DB + DS). Calculation is made with a 20 inch slide rule.

pH 5.4				pH 5.7				pH 6.0				pH 6.8			
$a-x$ obs.	k	b	y when $b = 0.45$	$a-x$ obs.	k	b	y when $b = 0.47$	$a-x$ obs.	k	b	y when $b = 0.59$	$a-x$ obs.	k	b	y when $b = 0.73$
M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5
4.83	0.215	0.42	2.17	4.83	0.215	0.42	2.27	5.59	0.152	0.59	3.30	6.21	0.107	0.71	4.55
3.05	0.208	0.44	1.37	3.28	0.192	0.48	1.54	4.00	0.148	0.60	2.36	4.83	0.107	0.71	3.53
2.15	0.189	0.49	0.97	2.31	0.179	0.52	1.09	2.76	0.153	0.59	1.63	4.14	0.094	0.75	3.02
								1.40	0.151	0.59	0.83	2.76	0.092	0.75	2.02
Average....		0.45				0.47				0.59				0.73	

pH 7.5				pH 7.8				pH 8.2			
$a-x$ obs.	k	b	y when $b = 0.84$	$a-x$ obs.	k	b	y when $b = 0.88$	$a-x$ obs.	k	b	y when $b = 0.93$
M × 10^5			M × 10^5	M × 10^5			M × 10^5	M × 10^5			M × 10^5
6.14	0.055	0.85	5.16	6.56	0.041	0.89	5.78	6.90	0.030	0.92	6.42
5.00	0.067	0.82	4.20	5.87	0.043	0.89	5.16	6.80	0.023	0.94	6.32
3.80	0.064	0.83	3.19	3.80	0.046	0.88	3.35	5.52	0.023	0.94	5.14
3.28	0.055	0.85	2.76	2.59	0.049	0.87	2.27	4.49	0.025	0.93	4.18
Average.....		0.84				0.88				0.93	

0.3. Extrapolation has been attempted by various methods but with such a curve it is very difficult to obtain any reliable result. We may assume, however, that we are not too far from the true value if we take the maximum value of k to be 0.37.

If we solve for the values of b in the above equation, we find that they remain fairly constant for each external pH value, but they increase with an increase in the external pH value as shown in Table II. It may be stated here that the values of b are the same whether $a - x$ represents the "total dye" or DB.

Knowing the values of b and $a - x$, we may calculate the values of y by means of the equation: $y = b\,(a - x)$ for any value of $a - x$ as shown in Table II.

In calculating the values of y, the observed values of $a - x$ (Table II) representing the "total dye" in the sap are used for convenience. Since we are interested primarily in the relative values of y, such values will give us the desired information. It is needless to state that if the values of DB in the sap were used instead of those of the total dye (DB plus DS), the values of y would be considerably lower than those given in Table II, but the ratio of one value of y to another would remain unchanged.

At each external pH value the values of y are found to increase with increase in the value of $a - x$. If we take a fixed value of $a - x$ and compare the values of y at different pH values, we find that the value of y increases with an increase in the external pH value.

In order to bring out clearly the effect of y on the velocity constant of exit we may return to the equation on page 90

$$k = k_1 - k_1 b$$

and substitute the value $b = \dfrac{y}{a - x}$ (see page 88). We then have

$$k = k_1 - \frac{k_1 y}{a - x}$$

It may be added here that the values of k are the same whether $a - x$ represents the "total dye" or DB.

Let us now consider the relation of y to the per cent[14] of DB in

[14] The discussion of the apparent dissociation constant is given in detail in the paper referred to in Foot-note 3.

the film (as determined by the pH of the external solution). We shall take for convenience the values of y where $a - x$ is 4.5×10^{-5} at different external pH values and take the percentage of DB as calculated from the distribution of DB between chloroform and water at different pH values of the external solution.

Let us first see if the values of y at different pH values are proportional to the values in per cent of DB obtained from the dissociation[3] curve of the dye (which gives the DB in per cent calculated from the data obtained by the experiments on the distribution of the dye between chloroform and water). If we take for convenience the value 4.5×10^{-5} M for $a - x$ and find the value of y at pH 7.8 at which pH value 20 per cent of the dye is in the form of DB (according to the dissociation curve) we are able to calculate the values of DB in per cent on the basis of the values of y at other pH values (since we know the values of y for this fixed value of $a - x$), by the following equation.

$$\frac{y_1}{y_2} = \frac{m_1}{m_2}$$

when y_1 = the value of y at pH 7.8 = 4×10^{-5} M.
y_2 = the value of y at another pH value, say pH 7.5 = 3.8×10^{-5} M.
m_1 = 20 per cent.
m_2 = the DB in per cent at pH 7.5.
By substituting we get

$$\frac{4 \times 10^{-5}}{3.8 \times 10^{-5}} = \frac{20}{m_2}$$

$$m_2 = 19 \text{ per cent}$$

Where the values of DB are thus obtained for different pH values, they are found to be higher than the values of DB of the dissociation curve.

Since the values of y do not appear to be directly proportional to the values of DB in the dissociation curve, we may look for another relationship. If we plot the values of $\frac{\text{per cent DB in external film}}{y}$ (at varying pH values of the external solution) against the per cent of DB in the film (z), we get a line which is fairly straight, as shown

in Fig. 4. This indicates a relation corresponding to Langmuir's[15] equation for adsorption,

$$y = \frac{m\,n\,z}{1 + zm}$$

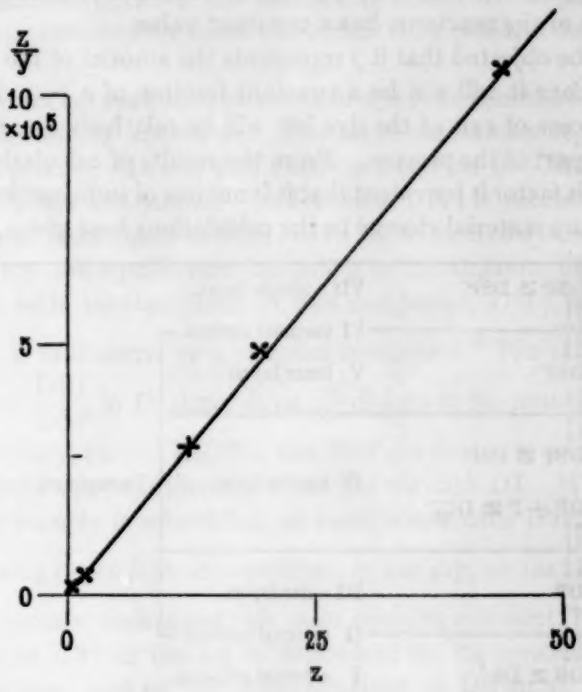

FIG. 4. Graph showing that Langmuir's equation $y = \frac{mnz}{1 + mz}$ (in which z = DB) may be applied to the process of the exit of the dye since the graph is a straight line. Ordinates represent $\frac{z}{y}$, and the abscissæ represent z.

where y is the substance adsorbed by a fixed concentration of an adsorbent, z is the concentration of the solution at equilibrium, and m and n are constants.

This might be regarded as indicating that the velocity constant (k) of the exit of the dye depends on the value of y which represents the

[15] Langmuir, I., *J. Am. Chem. Soc.*, 1918, xl, 1368.

amount of DB adsorbed by the protoplasmic surface from the film of external solution just outside the surface. But the applicability of this equation does not necessarily mean that we have to do with adsorption. For example, as Hitchcock[16] has pointed out, a similar relation applies if we have to do with a reversible chemical reaction where one of the reactants has a constant value.

It may be objected that if y represents the amount of DB adsorbed at the surface it will not be a constant fraction of $a - x$ during the entire process of exit of the dye but will be relatively greater during the latter part of the process. From the results of calculations which neglect this factor it is evident that it is not one of sufficient importance to effect any material change in the calculations here given.

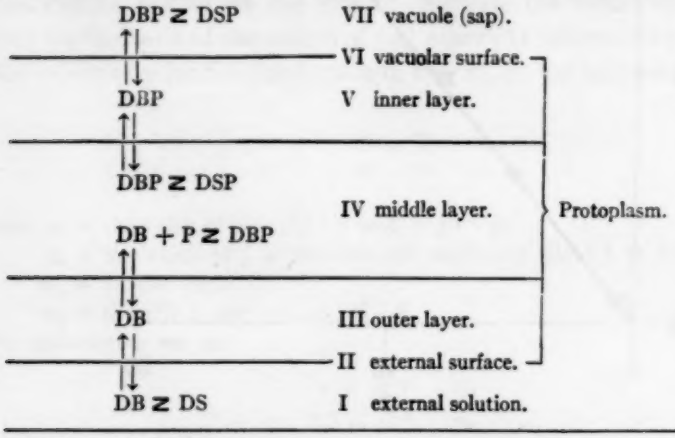

Diagram B. The cell wall is omitted for convenience.

If the surface forces constrain the molecules of DB so that they are not free to diffuse we meet with a difficulty. This difficulty would disappear if a constant fraction of the molecules is so constrained since that would merely lead us to divide the values of y by a constant factor.

The writer does not wish to lay any emphasis upon the fact that adsorption might possibly explain the relations observed but prefers to give the formula as a purely empirical one leaving the interpretation to future research.

[16] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–26, viii, 61.

The preceding discussion of diffusion applies whether DB is in the form of undissociated molecules or ions.

Let us now consider the hypothesis that the rate of penetration and likewise of exit of dye from the vacuole is controlled by a chemical combination between DB and a constituent of the protoplasm. For this purpose we may modify Diagram A to conform to Diagram B or C.

Equilibrium between the two forms of dye is designated by the sign ⇄ and diffusion by arrows ⇄. The entire mechanism represents a reversible process. Let us first take up Diagram B. DB can pass through III but DS cannot. As DB enters IV, it reacts with P, a protoplasmic constituent to form DBP. This form of dye compound, DBP, enters into equilibrium (according to the apparent dissociation constant) with another form of dye compound, DSP, which may represent a tautomere, or a complex compound. The ratio of $\frac{DB}{DS}$ and that of $\frac{DBP}{DSP}$ in IV depends on conditions in the protoplasm (pH value, solubility, etc.). DB, DS, and DSP are unable to pass through V, while DBP can pass through V but not through III. When DBP enters the vacuole it establishes an equilibrium with DSP, the ratio of $\frac{DBP}{DSP}$ being dependent on conditions in the sap, so that as long as the latter remain unchanged this ratio remains constant. The concentration of DBP in the sap is dependent on its concentration in the protoplasm, and on the concentrations of DB in all the parts described in the diagram. Thus, if the concentration of DB in III diminishes by its exit from III to I, then DB in IV decreases by its exit from IV to III, thus resulting in a decrease in DBP which in turn causes a corresponding amount of DBP to diffuse out from VII to IV.

We may consider a cell of *Nitella* in a solution as representing a heterogeneous system consisting of at least three phases: (1) the external solution, (2) the protoplasmic layer, and (3) the sap in the vacuole. If we venture to suppose that the protoplasm has non-aqueous layers at its outer and vacuolar surfaces, we shall consider the system to be composed of at least five phases.

The relation of the reaction DB + P ⇄ DBP in the protoplasm to the DB in the external solution may be made clearer if we consider the

hydrolysis[17] of an ester in hydrochloric acid when the ester is distributed between HCl and benzene. As fast as the ester is hydrolyzed in hydrochloric acid, more ester passes in from the benzene. The rate of hydrolysis is controlled by the distribution coefficient, C, of ester between hydrochloric acid and benzene, since the lower the value of C the less ester diffuses from benzene to the hydrochloric acid in a given time. The equation for this process resembles that for a unimolecular reaction in a homogeneous system. The only difference is that this equation contains the correction for the partition coefficient C.

Thus in the case of the reaction DB + P ⇄ DBP, the rate may be assumed to be dependent on the amount of DB that passes into the protoplasm. If the concentration of DB in the external solution is raised, more DB will enter the protoplasm in a given time, and this will increase the rate of reaction. Exit of the dye from the protoplasm may also be explained on this basis. If there is no DB outside the cell, DB will come out of the protoplasm, and with the decrease in the concentration of DB in the protoplasm, the reaction DBP → DB + P will proceed faster; DB thus formed will continue to come out until there is no DBP in the protoplasm. But if the DB which comes out is not at once removed, a certain amount will diffuse back into the protoplasm, so that in a given time the decrease in the concentration of DB in the protoplasm will be less than when there is no DB outside. This will correspondingly retard the process DBP → DB + P, and hence diminish the rate of exit of DB.

So far as the relation of the reaction DB + P → DBP in the protoplasm to the DBP in the sap is concerned, the same explanation will hold. With an increase in the concentration of DBP in the protoplasm more DBP will diffuse into the sap. If DBP in the protoplasm decreases, on the other hand, DBP will tend to come out of the vacuole into the protoplasm. The rate of the reaction will depend on the concentration of DBP in the protoplasm. If for example the concentration of DBP in the sap is increased, causing a decrease in the amount of DBP diffusing out of the protoplasm into the vacuole, the concentration of DBP in the protoplasm will increase. This increase will retard the reaction DB + P → DBP. Thus the rate of reaction DB + P → DBP

[17] Goldschmidt, H., and Messerschmidt, A., *Z. physik. Chem.*, 1899, xxxi, 235.

is controlled by the concentration of DB and DBP in the protoplasm. Increase in DB will hasten the reaction DB + P → DBP, while increase in DBP will retard it. The concentration of DB in the protoplasm depends on the amount of DB that enters or goes out of the protoplasm at a given time, and hence on the velocity of diffusion of DB through II or III in the diagram. The concentration of DBP in the protoplasm depends on the amount of DBP that goes out of the protoplasm into the vacuole, and the amount of DBP that enters the protoplasm from the vacuole, at a given time, and hence the rate of diffusion of DBP through V or VI in the diagram, B. It must be added here that the concentrations of DB and DBP are obviously interdependent.

In view of the fact that the time curve for the hydrolysis of ester in hydrochloric acid, as described above, follows an equation similar to that of an irreversible unimolecular reaction in a homogeneous system, it is not surprising that we find in the case of penetration of dye into *Nitella* the unimolecular time curves for a homogeneous system.

Thus the analysis of the time curve of the exit of the dye may be made in this case by the use of the same equation as in the case of diffusion

$$\frac{dx}{dt} = k_1 (a - x) - (k_1 y)$$

where a denotes the concentration of DB at the start in the protoplasm, $a - x$ the amount left combined with protoplasm at time t, and y the amount of DB in the film of liquid just outside the external protoplasmic surface. The presence of DB in the film will cause some DB to diffuse back into the protoplasm, and thereby retard the decrease of DB in the protoplasm. This retards the rate of the reaction DBP → DB + P and hence it retards the exit of the dye from the vacuole.

What we actually measure is the concentration of the total dye (DBP and DSP) in the sap, and the value of $a - x$ is taken from the amount of the total dye in the sap at equilibrium. This method is justified since we are interested primarily in the relative values, and since we assume that the amount of the dye in the sap has a definite ratio to that of the dye in the protoplasm.

Another method of explanation is the following, as described in Diagram C.

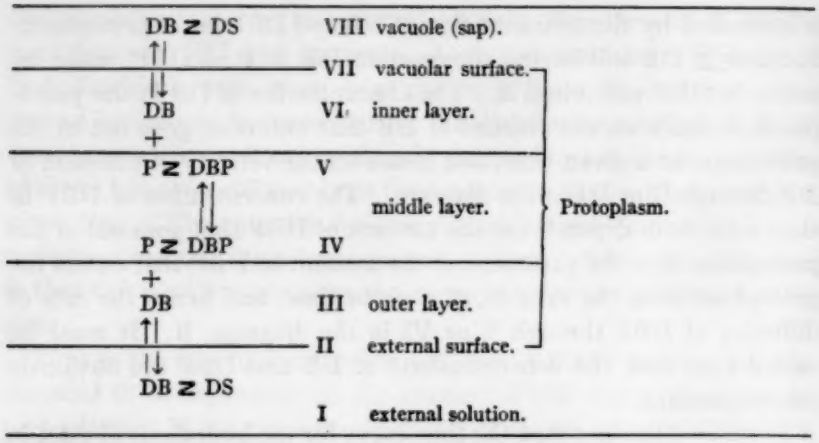

Diagram C. The cell wall is omitted for convenience.

DB can pass through III and VI. As DB enters at IV (the boundary between the outer layer and the middle layer) it combines with P of the protoplasm to form a complex compound DBP. DBP now diffuses from IV to V (the boundary between the middle layer and the inner layer) and DB is given off at V to VI. DB now diffuses into VIII. In I, II, VII, and VIII DB is in equilibrium with DS. The rate of DB + P → DBP is controlled by the concentration of DB and DBP in the protoplasm, and the concentrations of these substances are dependent on the amount diffusing in and out of the protoplasm in a given time. It is hardly necessary to undertake a detailed description of this diagram, as it closely resembles Diagram B. The only important differences are that the nature of the dye in the vacuole is not changed in this case, and that the reaction of DB + P → DBP in the protoplasm takes place at the boundaries IV and V. The latter may bring in complications to such an extent that we may have no justification for using an equation for a homogeneous reaction. Since so little is known in regard to this, the investigation of this question will be left to the future.

The experimental results thus far obtained do not show conclusively which one of the theories represents the mechanism. It may be possible that though there are reactions taking place between the dye and the protoplasmic constituents, the final result in both entrance of dye into and exit from the vacuole is dependent on the diffusion (see Section I) of the dye (see Foot-note 13).

A rough analogy to the passage of dye may be found in the case of entrance and exit of water into and from a reservoir, where the rate of inflow and outflow of the water depends on the conditions at the entrance and exit, and not on the conditions in the body of water between these two points.

V.

Rate of Exit When the pH Value of the Sap Is Changed.

The following experiments were carried out to determine if the theory thus proposed is supported by the observations on changes in the rate of the exit of the dye when the pH value of the sap is varied.

One lot of cells was placed in M/150 borate buffer solution at pH 8.5 containing 8.6×10^{-5} M cresyl blue and 0.005 M NH_4Cl; at the end of 5 minutes there was 8.6×10^{-5} M dye in the sap. The cells were then removed, wiped with a damp cloth, and placed in an M/150 phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes, the concentration of the dye in the sap was found to be 2.6×10^{-5} M.

A second lot of cells was placed in 0.005 M NH_4Cl at pH 8.5 M/150 borate buffers: at the end of 5 minutes the pH value of the sap had increased from pH 5.6 to 6.9. The pH value of the sap remained at 6.9 when such cells were placed in a buffer solution at pH 6.5 for 2 minutes.

A third lot of cells was placed in M/150 borate buffer solution at pH 8.5 containing 8.6×10^{-5} M cresyl blue: at the end of 45 seconds there was 8.6×10^{-5} M dye in the sap. The cells were now removed, wiped with a damp cloth, and placed in an M/150 phosphate buffer solution at pH 6.5 containing no dye. After 2 minutes the concentration of the dye in the sap was determined and was found to be 5.9×10^{-5} M.

In all cases the experiments were carried out at 25 $\pm$ 0.5°C., and the solutions were constantly stirred and changed.

From these experiments it may be concluded that the rate of the exit of the dye from the cell sap is increased by presence of NH_3 in the sap which increases the pH value of the sap. Whether this increase in the rate is due to the increase in the pH value of the sap, or to the possible presence of NH_3 and consequent increase in pH value in the protoplasmic layer, or to NH_3 adhering to the cell surface, the writer is at present unable to determine.

The above observation is in agreement with the theory since the increase in the pH value would increase the concentration of DB in the sap and hence increase the concentration gradient, but in view of the fact that no appreciable changes in the pH value of the sap may be brought about without an injury to the cells, such a conclusion must necessarily be made with reserve. Furthermore, the fact that the rate of penetration is decreased,[18] while that of the exit of the dye is increased, when NH_3 enters the sap, does not prove that the dye enters the cell only in the form DB. As already suggested by the writer, in case DS enters,[19] the rate of penetration[20] may very well be decreased by the competition between the DS and the aqueous NH_3 for the substances (*viz.* salt of proteins or weak acid) in the protoplasm which may take place as NH_3 and DS enter the cell. The presence of aqueous NH_3 then would hasten the exit of DS from the cell, if the affinity of NH_3 for the cell substance is greater than that of DS.

SUMMARY.

Experiments on the exit of brilliant cresyl blue from the living cells of *Nitella*, in solutions of varying external pH values containing no dye, confirm the theory that the relation of the dye in the sap to that in the external solution depends on the fact that the dye exists in two forms, one of which (DB) can pass through the protoplasm while the other (DS) passes only slightly. DB increases (by transformation of DS to DB) with an increase in the pH value, and is soluble in substances like chloroform and benzene. DS increases with decrease in pH value and is insoluble (or nearly so) in chloroform and benzene.

The rate of exit of the dye increases as the external pH value decreases. This may be explained on the ground that DB as it comes out of the cell is partly changed to DS, the amount transformed increasing as the pH value decreases.

The rate of exit of the dye is increased when the pH value of the sap is increased by penetration of NH_3.

[18] McCutcheon and Lucke (see Foot-note 9) believe that the decrease in the rate of penetration of a basic dye into *Nitella* with an increase in the pH value of the cell sap is a direct disproof of the theory that the dye combines with a protein in the cell.

[19] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 235.

[20] When the pH value of the sap is decreased by an entrance of acetic acid the rate of penetration of dye is either increased or decreased, depending on the condition (probably of the protoplasm) of the cell.

FURTHER STUDIES ON THE INHIBITION OF CYPRIDINA LUMINESCENCE BY LIGHT, WITH SOME OBSERVATIONS ON METHYLENE BLUE.

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In a previous paper (1924-25) I have shown that a filtered luminescent mixture of *Cypridina* luciferin and luciferase has its luminescence suppressed (inhibited) by light from a carbon arc in 2 or 3 seconds with 15,000 foot candles illumination. The action of the light is upon the luciferin and not upon the luciferase. I have reported the suppression as partially reversible, the luminescence returning slightly in the dark, but am now inclined to regard this effect as apparent, due to better dark adaptation of the eyes. The inhibiting wave-lengths are in the blue-violet region, $\lambda = .46\mu$ to $\lambda = .38\mu$. Consequently one finds no suppression of luminescence by red, orange, yellow, or green light even after prolonged exposure.

The present paper deals with three further aspects of inhibition of luminescence by light, namely (1) photodynamic action of dyes; (2) influence of oxygen; (3) influence of H ion concentration on the inhibition.

1. *Photodynamic Action of Dyes.*—As the dye sensitization of many photochemical reactions is well known—notably that of the photographic plate by dicyanin, pinacyanol, erythrosin, etc., and the photodynamic action of acridine, eosin, etc., on living tissues, enzymes, and antibodies— it is not surprising to find a similar effect of dyes on the inhibitory action of light on *Cypridina* luminescence.

I have said that inhibition is brought about by blue (.46 to .38μ) light but not by red, orange, yellow, or green light. However, if we add to the luciferin–luciferase mixture one of a number of dyes, then we find that red, orange, yellow, or green light will inhibit the luminescence in a few seconds. The wave-length of light which will inhibit

Cypridina luminescence in the presence of sensitizing dye depends, of course, on the position of the absorption band of the dye, only those wave-lengths inhibiting which are absorbed. The converse is not necessarily true, that if a dye possesses an absorption band it is a photosensitizer with respect to that light. There may be or may not be sensitization.

A number of dyes have been tested by a student of mine, Mr. A. Hunsberger, Jr. The method is this. In a dark room, light from a carbon arc in a dark house passes through 6 cm. of water and is condensed to a small beam which strikes the middle portion of a narrow test-tube containing the luminescent mixture of *Cypridina* luciferin and luciferase. The light beam can be colored by light filters and cut off instantly by a photographic shutter. The luminescence of the exposed area of the test-tube is then compared with the non-exposed regions above and below.

Wratten gelatin filters were used to obtain light of a known range of wave-lengths. As the percentage transmission of the filters varies, some being much denser than others, it is impossible to select filters that will permit equal amounts of nearly monochromatic light to pass. In fact I have selected filters of high transmission which begin to absorb strongly at some definite wave-length (No. 15, 22, 29, 88) or those with broad transmission bands (No. 61) rather than the denser monochromatic filters, in order that the exposure need not be too long. 15 seconds was selected as a convenient time.

The filters are:

No. 61 green; over 10 per cent transmission .50 to .57μ.
" 15 yellow; " 10 " " " .52 " .70μ and beyond.
" 22 orange; " 10 " " " .55 " .70μ " "
" 29 red; " 10 " " " .62 " .70μ " "
" 88 infra-red; no visible transmitted except 5 per cent at .70μ.

The dyes tested are given in Table I.

Allowing for the unequal transmission of the filters and the widening of the absorption bands of the dye with increase in concentration, there is undoubtedly an agreement with the rule that the dyes sensitize only for that wave-length of light which they absorb.

It can also be shown that the inhibiting action of green light in pres-

ence of eosin is upon the luciferin and not the luciferase, just as in the case of violet wave-lengths acting without sensitizer.

I think we may predict with fair certainty that Ctenophores, the inhibition of whose luminescence by light is so well known, will also be sensitized by photodynamic dyes.

2. *Influence of Oxygen.*—It must be recalled that luminescence only occurs if luciferin, luciferase, and oxygen are together in solution, but that luciferin will oxidize without luminescence if luciferase is absent.

It seems most probable, therefore, that light acts by causing rapid

TABLE I.

Dye.	Structure.	Absorption in weak and stronger sol.	Photosensitization in 15 sec.
		μμ	
Fluoresceine.		475–502 450–505	Negative.
Eosin.	K tetrabromfluoresceine.	500–530 480–540	Green, yellow.
Erythrosin.	K or Na tetraiodofluoresceine.	500–530 480–560	" "
Rose bengale.	K or Na 4 iodo 2 chlorfluoresceine.	530–550 480–560	" "
Cyanosin.	K methyl ether of 4 Br 2 Cl fluoresceine.	500–545	" "
Acridine or aniline red E 103.		480–530 430–570	" "
Methylene blue.	Tetramethyl thionin HCl.	650–680 560–680	Yellow, orange, red.

oxidation of luciferin without luminescence. Consequently the area exposed to light, of a luminescent mixture of luciferin and luciferase in a test-tube, will not luminesce so brightly because some of the luciferin has been photochemically oxidized. This view can be tested by completely exposing luciferin solutions to light in absence of oxygen (by evacuation or bubbling of pure hydrogen), with a control tube illuminated in presence of oxygen, and then mixing both tubes with luciferase. The luciferin exposed in absence of oxygen gives a bright light, while the control in presence of oxygen gives no luminescence or a very faint one. There is no inhibition in absence of oxygen.

I find also that the photosensitive dyes will not sensitize the inhibition of luminescence from luciferin in green or yellow light in absence of oxygen. In this respect the phenomenon agrees with photodynamic dye action in tissues. There is no poisonous action of eosin on enzymes or organisms in light in absence of oxygen (von Tappeiner, 1909).

The *dye* simply acts by making wave-lengths effective which would not be effective in its absence. The *light* acts by rapidly oxidizing luciferin.

The manner in which the photosensitizers make wave-lengths photochemically effective is not well understood. Perhaps the first question to be asked is whether the sensitizer undergoes any change. We may suppose the eosin to oxidize the luciferin in presence of light, itself undergoing reduction to a leuco body. Accordingly I have exposed mixtures of luciferin and eosin to white light (8600 foot candles) and also to green light, for from 4 to 7 minutes, but have never observed any indication of the decolorization of eosin, although it is known that eosin is affected by light (Gros, 1901). There is also no indication of the decolorization of methylene blue and luciferin exposed to white light (8600 foot candles) for 4 minutes, although the oxidative action of the light on luciferin is greatly increased by the presence of these dyes. If any change occurs in the dye it involves no color change or is momentary. Under proper conditions, however, methylene blue is affected by light, as described below.

3. *Influence of H Ion Concentration.*—I have often compared luciferin to leuco methylene blue and its oxidation to the oxidation of leuco methylene blue with formation of the blue dye. Expressed as a reaction the change would be

MH_2 (leuco methylene blue or methylene white) $\rightleftarrows$ M(methylene blue) + H_2

LH_2 (luciferin $\rightleftarrows$ L (oxyluciferin) + H_2.

$$H_2 + O = H_2O$$

In view of the effect of light on luciferin oxidation it is interesting to note that methylene white oxidation is affected by light also. This was observed by Clark (1925), and the effect can be very nicely seen by reducing methylene blue with Zn dust and dilute acid, pouring the

colorless solution into a narrow test-tube and exposing the middle portion of the colorless solution to the condensed beam from a carbon arc lamp, first passing the beam through a water layer to remove its heat. Blueing will occur in the illuminated region in a few seconds. Oxygen is of course present in this experiment, but I can confirm Clark's observation that blueing will occur *in absence of oxygen* after reduction by platinized asbestos and hydrogen. *However, the solution must be acid.* After removal of oxygen and reduction by $Na_2S_2O_4$, blueing of methylene white in light will also occur, provided the solution is acid enough and that not too much $Na_2S_2O_4$ has been added. Neutral and alkaline solutions will not turn blue in light under the same conditions. There seems to be a shift in equilibrium of the methylene blue $\rightleftharpoons$ methylene white system toward the side of oxidation in the light.

If methylene blue is reduced by Pt asbestos and hydrogen in two tubes, one of M/50 HCl, the other of M/50 NaOH, and the tubes shaken slightly to dissolve a little oxygen it can be easily observed that the methylene white oxidizes much more quickly in the alkaline tube, which becomes blue as compared to the acid tube, only faintly bluish—a well known phenomenon. On now exposing the two tubes to a beam of light, there results a blue band in the acid medium and a colorless band in the alkaline medium. We have acceleration of oxidation in acid and of reduction in alkali in light. I have observed the same thing when NH_4SH or H_2S is used as reducing agent and also when $Na_2S_2O_4$ is employed.

If just enough $Na_2S_2O_4$ is added to decolorize methylene blue in M/50 HCl and the colorless tube is exposed to light, blueing will occur; but if a little more $Na_2S_2O_4$ is added no blue band occurs in light. However, upon shaking with air until some of the $Na_2S_2O_4$ is removed by oxidation, a blue band now appears in light. Reducing with $Na_2S_2O_4$ in an alkaline medium (Clark and Lubs buffer, M/20 H_3BO_3, KCl, NaOH, pH = 10) and exposing to light, we observe no change; but if shaken with air till partly blue and then exposing to light, a colorless band appears.

This colorless substance is methylene white and not a colorless oxidation product of methylene blue, because by thorough shaking with air the blue color will return again completely.

We may sum up the behavior of methylene blue in light as follows: Methylene white in presence of reducing agents will turn blue in absence of oxygen if the solution is acid enough but not in neutral or alkaline solutions. In presence of some oxygen and reducing agent,[1] acidity favors the change to blue (oxidation) while alkalinity favors the change to colorless (reduction). Without reducing agent, methylene blue will be rendered colorless by light slowly in fairly alkaline solution (M/50 NaOH) but not in M/10 Na_2HPO_4 (pH = 9) or in distilled water.

Indigo carmine does not behave like methylene blue under the influence of illumination.

If the luciferin–oxyluciferin system is to behave like methylene blue, we should expect inhibition of luminescence in light in acid medium containing some oxygen and reducing agent[1] (like $Na_2S_2O_4$). Under these conditions oxidation to oxyluciferin would be favored and less luciferin remain to luminesce with luciferase. Hence a dark band should appear in a luminescent tube after illumination. On the other hand, in alkaline medium a luminescent band should appear after illumination, since reduction would be favored and more luciferin accumulate in the previously illuminated region.

However, I have been unable to observe a more luminescent band after illumination in solutions of any reaction. If luciferin is prepared in a series of buffer solutions and a little luciferase added we get the following results in light-exposed and dark regions of the tube. No reducing agent is present.

Buffer solution and pH.	Luminescence in dark.	Luminescence in light.
M/20 K H phthalate = 4.	Very faint.	Very slowly inhibited.
M/20 K H phthalate, NaOH = 5.6	Faint.	" " "
Sea water = 8.	Good.	Rapid inhibition.
M/20 H_3BO_3, KCl, NaOH = 9.	Faint, fading quickly.	" "
M/20 " " " = 10.	" " "	" "

It will be noted that inhibition, indicating oxidation of luciferin, always occurs no matter what the reaction, acid or alkaline.

If $Na_2S_2O_4$ is now added to the above tubes to remove the oxygen

[1] Or its oxidation product.

the luminescence disappears and exposure to light in absence of oxygen never causes luminescence to return. If the tubes are shaken slightly to dissolve oxygen, luminescence will return and then exposure to light gives the same results as recorded in the table in the absence of any reducing agent. Light exposure always results in inhibition which is more rapid the more alkaline the medium and is also more rapid in presence of the $Na_2S_2O_4$ (or its oxidation products) than previously. It is as if the oxidation products of $Na_2S_2O_4$ accelerated the effects of light, as they do in the case of methylene blue. I have never observed a more luminescent band in the region previously exposed to light.

We see that the behavior of luciferin in light is only in part similar to that of methylene blue. One always obtains acceleration of oxidation of luciferin by light and not acceleration of reduction under the same conditions (alkaline medium) necessary for the phenomenon in methylene blue. Perhaps it is pushing the analogy too far to expect that the methylene white—methylene blue and the luciferin–oxyluciferin systems will behave in exactly the same way after exposure to light.

SUMMARY.

1. Eosin, erythrosin, rose bengale, cyanosin, acridine, and methylene blue act photodynamically on the luminescence of a *Cypridina* luciferin–luciferase solution. In presence of these dyes inhibition of luminescence, which without the dye occurs only in blue-violet light, takes place in green, yellow, orange, or red light, depending on the position of the absorption bands of the dye.

2. Inhibition of *Cypridina* luminescence without photosensitive dye in blue-violet light, or with photosensitive dye in longer wave-lengths, does not occur in absence of oxygen. Light acts by accelerating the oxidation of luciferin without luminescence. Eosin or methylene blue act by making longer wave-lengths effective, but there is no evidence that these dyes become reduced in the process.

3. The luciferin–oxyluciferin system is similar to the methylene white–methylene blue system in many ways but not exactly similar in respect to photochemical change. Oxidation of the dye is favored in acid solution, reduction in alkaline solution. However, oxidation

of luciferin is favored in all pH ranges from 4 to 10 but is much more rapid in alkaline solution, either in light or darkness. There is no evidence that reduction of oxyluciferin is favored in alkaline solution. Clark's observation that oxidation (blueing) of methylene white occurs in complete absence of oxygen has been confirmed for acid solutions. I observed no blueing in light in alkaline solution.

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TEMPERATURE CHARACTERISTICS FOR DURATION OF AN INSTAR IN CLADOCERANS.

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I.

The analysis of growth phenomena through determinations of their relations to temperature requires measurements of the rates of development within stages morphologically well defined and exhibited by individuals genetically uniform and comparable. The duration of an adult instar in Cladocera parthenogenetically produced within a single clone is in several respects a very suitable object for such measurements. It is not altogether clear, however, whether the incidence of ecdysis is directly determined by the growth of the female, or by the full development of her young which ecdysis liberates. It is proposed to investigate this point more fully in subsequent experiments, but the latter interpretation is probably the correct one, for mothers about to release young, if transferred to a considerably higher temperature, will release the young properly but they themselves become caught in the moult which is being cast. In other words the young, at the higher temperature, have reached full development and have apparently caused the mother to moult before the carapace was in proper condition to be cast. For the present it is sufficient to note that the period of growth or development utilized for measurement bears relations to temperature of a kind very closely comparable with those shown by relatively simple vital processes (Crozier, 1924–25, *a*, *b*), and obtained likewise for the velocity of development within a clearly delimited stage in *Drosophila* (Bliss, 1925–26).

Comparison of several species of cladocerans reared in the laboratory for many generations under similar conditions should reveal spe-

cific differences, should such be present, and might be taken to suggest a means for the physiological comparison of related forms.

Three species of Cladocera were employed. Two of these, *Moina macrocopa* and *Simocephalus serrulatus*, belong to the family Daphnidæ; the third species, *Pseudosida bidentata*, belongs to the family Sididæ. A few individuals of *M. macrocopa* and of *P. bidentata* were sent me by Dr. A. M. Banta, both forms having been reared parthenogenetically for many generations in his laboratory. *M. macrocopa* was originally collected near Cold Spring Harbor, Long Island, and it occurs there in small exposed ponds from April through October. The population in the ponds increases rapidly to a maximum in June and the form is found in slightly diminished numbers until the middle of October. The clone of *P. bidentata* that was used was started from individuals collected in Florida in February; I have not taken this form in the north. *S. serrulatus* was collected near Cambridge, Massachusetts, early in October. In general it is a species that is quite wide spread and it may also be taken during any month of the year, usually reaching large numbers towards the last half of April and the first of May, continuing with few individuals during July and August, and reaching a maximum in October.

II.

In the laboratory, reproduction in all species was exclusively parthenogenetic, starting from one female and thus insuring material genotypically identical throughout. The stock cultures were reared at room temperature in an especially devised culture medium (Banta, 1921). The animals used in any one experiment were usually the first or second generation descendants of one individual. Such animals were reared with ample food, so that they would produce average sized to large broods, brood size being a good indication of the vigor of the stock. For precise work it is necessary to study a stage of development having sharply marked beginning and end. In this case one adult instar, usually the second, was chosen as the period to be measured. The successive instars of a given animal, when reared at the same temperature, do not show any appreciable differences in length. The beginning of an adult instar is marked by the release of active young daphnids from the brood chamber of the mother, and the

end of the instar is marked by the release of the succeeding brood of young. In *M. macrocopa* the release of the young, the moulting of the mother, and the passing of a new clutch of eggs to the brood chamber occupies from 2 to 6 minutes at room temperature. The time for this series of events is slightly more variable in *P. bidentata*, but not so variable as in *S. serrulatus* in which the time from release of young to egg laying varies from a few minutes to half an hour in extreme cases.

As the adult females to be tested were nearing the end of an instar they were transferred to individual bottles and placed in constant temperature cabinets. A bottle of food was placed beside a bottle containing a female. The termination of an adult instar, that is, the appearance of young in the bottle, is foreshadowed by the darkening and coalescing of the eye rudiments of the embryos. Observation of the mothers was made at frequent intervals (15 minutes to an hour, depending on the temperature), and the time of the release of the young noted. The mother was transferred to the adjacent bottle and allowed to remain there until the next brood of young was released. When it was impossible to watch for the termination of the instar in question at intervals of an hour or less, those mothers that probably would soon liberate young were examined microscopically and an estimation made of the probable time of release of her young (based upon the condition of the eye pigment of the embryos). This estimated time was further checked by observing the developing eggs of the next brood and noting the stage of segmentation of the egg. If the elapsed time was too long the animal was of course discarded.

Four temperature cabinets were used, in each of which the temperature to be maintained could be raised or lowered at will. The cabinets were heated by carbon filament bulbs controlled by a mercury thermoregulator. The different regions (shelves) of the cabinet varied in temperature depending on the distance from the heating unit, but the temperature of each bottle was taken directly. The extreme variation within a given bottle during an experiment was $\pm$ 0.5°C. Slightly different rates of general metabolism are found for the females producing male broods and those producing female broods (Banta and Brown, 1924–25). This has been determined for *M. macrocopa;* the male broods are released on the average later (half an hour, more or

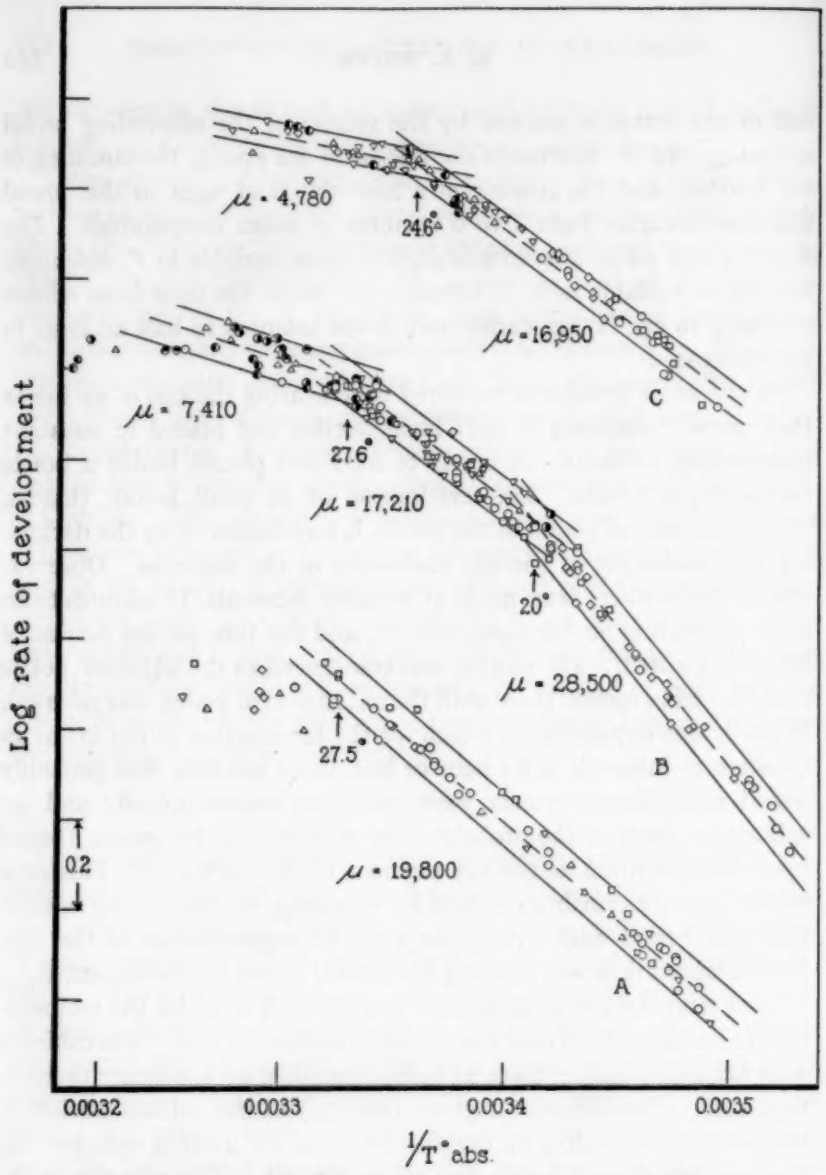

FIG. 1. Graphs for rates of development plotted against reciprocals of the absolute temperatures. *A*, *Pseudosida bidentata*. *B*, *Moina macrocopa*. *C*, *Simocephalus serrulatus*. Each point represents an individual animal and the different symbols in each graph denote a single experiment. The values of μ are given opposite the segments of the graphs, and the critical points are indicated by arrows, with the centigrade temperatures given for these points. (Rate of development = 10,000 ÷ time in minutes; one unit on log scale = 0.2.)

less) than the female broods. It has not been possible to employ numbers of animals sufficient to remove this source of variation, which would at most amount to between 1 and 2 per cent of the total observed time.

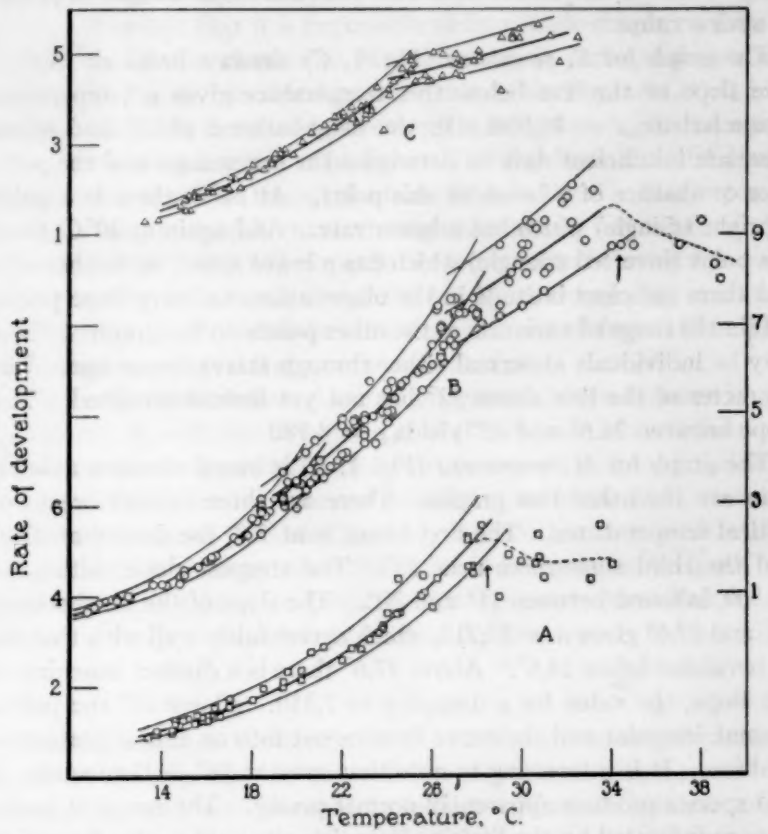

FIG. 2. *A, P. bidentata. B, M. macrocopa. C, S. serrulatus.* The rates of development are plotted against centrigrade temperatures, and the points are entered without distinguishing individual experiments. The lighter lines and the heavier lines are transposed from those in Fig. 1.

III.

Fig. 1 gives the graphs for the rates of development in the three species. The logarithm of the rate is plotted against the reciprocal

of the absolute temperature. Each point on the graph represents an individual animal and the different symbols in each graph denote a single experiment. The experiments were conducted at different times between January and May of the same year to examine the possibility of seasonal rhythm. It is clear that there is no secular drift due to such a cause.

The graph for *S. serrulatus* (Fig. 1, *C*) shows a break at 24.6°C. The slope of the line below this temperature gives a temperature characteristic $\mu = 16{,}950$. In the neighborhood of 15° and below there are insufficient data to determine the exact slope and the presence or absence of a break at this point. At 24°C. there is a point (upright triangle) which has a lower rate. And again at 29°C. there is a point (inverted triangle) which has a lower rate. In neither case was there sufficient latitude in the observations to bring these points within the range of variation of the other points on the graph. These may be individuals abnormal either through starvation or age. The character of the line above 32° has not yet been determined. The slope between 24.6° and 32° yields $\mu = 4{,}780$.

The graph for *M. macrocopa* (Fig. 1, *B*) is based on more animals than are the other two graphs. There are three evident breaks or critical temperatures. The first break is at 20°, the second at 27.6° and the third somewhere near 33°. The steepest slope, with $\mu = 28{,}500$, is found between 11° and 20°. The slope of the line between 20° and 27.6° gives $\mu = 17{,}210$, which agrees fairly well with that for *S. serrulatus* below 24.6°. Above 27.6° there is a distinct lessening of the slope, the value for μ dropping to 7,410. Above 33° the points become irregular and the curve flattens out into an almost horizontal position. It is interesting to note that even at 38° + the females of this species produce apparently normal young. The range of variation, as indicated by the limiting lines, is quite uniform for the different parts of the graph, being slightly greater above 27°; this may be due to the faster rate of development and the correspondingly decreased accuracy of the observations.

The graph for *P. bidentata* (Fig. 1, *A*) is strikingly different from the other two. This graph consists of one straight band, having the value of $\mu = 19{,}800$, extending from 14° to 27.5°. Beyond 28° the points scatter and the curve assumes a position approximately parallel

to the temperature axis. There is thus but one break, at 27.5°, in the graph for *P. bidentata*.

Inspection of graphs such as that for *M. macrocopa* in Fig. 1 may lead to the superficial suggestion that it is preferable or possible to draw through the plotted points a curve, rather than several straight lines. The fact that it is impossible to fit a single simple curve to the graphs for *S. serrulatus* and *M. macrocopa* is perhaps shown more clearly in Fig. 2. In this figure the rate of development, that is the reciprocal of the time taken to complete an instar, is plotted against centigrade temperature. The lighter limiting lines and the heavier lines in this figure are transposed from those in Fig. 1. It is impossible to fit a single smooth curve through the points for either of these species. The points fall respectively into two and three cusps (when the irregular individuals above 33° for *M. macrocopa* are excluded). The points for *P. bidentata* form a smooth curve from 14° to 27.5°, this whole range yielding a constant value of μ.

The range of variation of course increases with an increase in temperature. It will also be noticed that the latitude of variation for *S. serrulatus* is much smaller than for *M. macrocopa* and for *P. bidentata*, in spite of the fact that the end-points for an instar in *S. serrulatus* are more variable, intrinsically, than in the other two species. This would seem to indicate that the latitude of variation is specifically determined, but is not a property of the system controlled by the nature of the process which determines the temperature characteristic.

IV.

A comparison of the values for μ with similar values obtained for rates of development in other arthropods shows some remarkable similarities, and may be taken to indicate a similar chemical control in the several instances. Bliss (1925–26) obtained a value for μ of 16,850 for the prepupal development of *Drosophila melanogaster*, from 16° to 25°. Crozier (1924–25, *b*), using data from Krogh, obtained a value of 16,850, 16° to 32°, for μ in O_2 utilization of *Tenebrio* pupæ, and Orr (1924–25) obtained a value for μ of 16,800, 1° to 15°, for the O_2 consumption in the prepupa and pupa of *Drosophila*. These values are of the same order as the 16,950 obtained for *S. serrulatus* between 15° and 25°, and the value 17,210 obtained for *M. macrocopa*

between 20° and 28°. The value of μ for *M. macrocopa* below 20° does not correspond to the value obtained by Bliss with *Drosophila* below 16°, but it may be of the same nature (28,500) as the value 27,000 obtained from Krogh's data by Crozier (1924–25, *b*), below 22° in *Tenebrio* pupæ, that for *Drosophila* egg and larva between 10° and 20° and for *Drosophila* pupa at 15° to 20°, both of which gave μ = 27,000 (Crozier, 1924–25, *b;* data from Loeb and Northrop). For *M. macrocopa* the temperature range 28° to 33° yields μ = 7,410, which compares favorably with the value obtained by Bliss for prepupal development in *Drosophila* between 25° and 30° (*i.e.*, 7,100). Also it might be noted that the distribution of the temperature characteristics, with the exception of 4,780 for the upper portion of the graph for *S. serrulatus*, corresponds to peaks in the frequency graph for temperature characteristics given by Crozier (1925–26, *d*).

The breaks in Fig. 1, when rounded off to the nearest whole degree, are located at 15° (?), 20°, 25°, 28°, 30°, and 33°. Crozier has pointed out (Crozier, 1925–26, *c*) that such critical points are usually found in the neighborhood of 4.5°, 9°, 15°, 20°, 25°, 27°, and 30°. Setchell (1925) has pointed out that aquatic and land plants give definite points of critical temperature for anthesis, these points being at approximately regular intervals of 5 degrees from 5° to 30°. The critical points obtaining for the three species of Cladocera thus agree in a rather remarkable way with those found for vital phenomena in general.

A comparison of the three species of Cladocera used in these experiments shows some points of possible significance. The temperatures for *S. serrulatus* above which rate of development is relatively slower, *i.e.* lower value for μ, are 15° (?), 25°, and 30°, while the corresponding temperatures for *M. macrocopa* are 20°, 28°, and 33°. Thus *M. macrocopa* is, upon the basis of rate of development at least, enabled to take advantage of increasing temperature by producing broods in quicker succession than *S. serrulatus*. And conversely, on a lowering temperature, *M. macrocopa* is relatively more slowed up. As previously stated, *M. macrocopa* is typically a summer form while *S. serrulatus* occurs throughout the year but in greater abundance in the spring and autumn. *P. bidentata* cannot be compared so directly. This species maintains a constant μ = 19,800 from 13° to 28°. This

rate of increase with temperature is decidedly greater than that of the other two species above 20°. The distribution of *P. bidentata* is known to be southern and was studied during the winter, but its annual rhythm of abundance is not known at present.

SUMMARY.

1. The temperature characteristics for the rate of development during a well defined instar were determined for three species of Cladocera, and found to be of the same general magnitudes as those obtained for rates of development and of O_2 consumption in other arthropods.

2. Critical temperatures were found to occur at points most frequently critical in quite diverse vital phenomena as determined by abrupt changes in the relationship between rate and temperature.

3. A suggestion is made that, since the values of μ and the positions of critical temperatures obtained for the different species are not the same, some relation may exist between the occurrence of these forms in nature and their relative rates of development as controlled by temperatures.

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THE INFLUENCE OF LIGHT, TEMPERATURE, AND OTHER CONDITIONS ON THE ABILITY OF NITELLA CELLS TO CONCENTRATE HALOGENS IN THE CELL SAP.

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In connection with a general investigation of the absorption and utilization of mineral elements by plants, various observations on the cells of *Nitella clavata* were previously reported.[1,2] The primary object of the experimentation is to gain some additional insight into the fundamental processes of absorption in their relation to the nutrition of higher plants. Special attention has, therefore, been given to dilute solutions, comparable to soil solutions, and to the intake of mineral elements by various types of plant cells from the point of view of growth and metabolism, rather than that of permeability *per se*. Certain phases of the general problem which were previously outlined have now received further study and the development of a number of new methods of procedure has made it possible to obtain much more extensive and satisfactory data than heretofore. Reference to the recent work of Osterhout and his colleagues on *Valonia* and *Nitella* will be deferred until later in the discussion.

In our earlier experiments on *Nitella* certain preliminary data were obtained on the absorption of bromine. This element seemed to offer promise of being useful in studying the phenomena of absorption, since it is non-toxic or practically so in low concentrations, and is not normally present in the cell sap of these plants. At first the estimation of bromine was made by a colorimetric method, but this was not found to be satisfactory for quantitative work and it did not appear

[1] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.
[2] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, vi, 47.

that any technique so far described for determining bromine met our particular requirements. One of the writers (P. L. Hibbard), therefore, devoted considerable effort to devising a method suited to the purpose. Eventually, it was found possible to determine bromine in the presence of chlorine or iodine with the use of very small quantities of cell sap (1 cc.) and within a reasonably short time. The analytical procedure and the magnitude of the error, when this method is applied to plant sap, are discussed elsewhere.[3] Considering the very minute absolute quantities dealt with, the accuracy of the results was better than might be anticipated. As a rule errors other than those connected with the analysis limited the interpretation of the data, except in those cases in which very small concentrations of bromine were present in the sap, when the percentage of error in the analytical data was unavoidably high. Nearly all analyses were made in duplicate and the experiments were also duplicated or repeated. It is believed that due care has been taken to limit the conclusions in accordance with the significance and consistency of the results, as will be further indicated in the discussion of the specific data. Fortunately, the effects we sought to demonstrate were of large relative magnitude so that they stand out quite clearly. At the present stage of development, it is extremely doubtful whether anything would be gained by any considerable refinement of the experiments, which might involve an almost prohibitive amount of labor.

Cell sap only slightly contaminated was obtained by the method of breaking individual plant cells in the manner previously described. Cells used for this purpose were very turgid and varied in length from $\frac{1}{2}$ to 3 inches. In addition to this method of obtaining sap, in a few experiments, sap was also prepared by expressing (by hand pressure) the masses of cells remaining after nearly all the large cells had been selected out. The sap prepared in this way and filtered is designated as "expressed sap" and that derived from individual cells as "cell sap." The different substances were present in the expressed sap in concentrations only about one-half those of the cell sap. Of course, there must have occurred a very appreciable dilution of the cell sap in the former case by water still adhering to the outer surfaces, even after

[3] Hibbard, P. L., *Ind. and Eng. Chem.*, 1926, xviii, 57.

shaking the mass of cells vigorously, but it is also possible that the many very small cells (not more than $\frac{1}{4}$ inch in length) contained sap of lower concentration than that of the sap in the large cells. Conclusions with regard to the general relations existing between the cell sap and the external medium would have been very similar if the expressed sap alone had been considered, but the results on the cell sap are obviously more definite and convincing, and, furthermore, the expressed sap cannot give an adequate idea of the extent to which certain elements may become concentrated in the vacuole.

In many of the experiments it was found convenient to use a general culture solution to which bromide was added as desired. This solution was buffered with phosphate and had the following approximate composition.

KH_2PO_4	5	milli-equivalents
$Ca(H_2PO_4)_2$	2	"
NaOH	6	"
pH	5.0–5.4.[4]	

During the course of experiments of extended duration the initial pH value of the culture solution usually increased by several tenths, whether as a result of selective absorption or because of the lime deposits frequently adhering to the outside surfaces of the cells. Solutions of the composition stated above were not found to show any apparent toxicity within the time of the experiments. Under favorable environmental conditions, masses of cells could be kept in such solutions over periods up to 2 months in as good a condition as in tap water. Bromides in a concentration of .005 molar were not toxic as far as could be observed and much higher concentrations produced only very slight injury, if any. Of course, in any solution, there occurs a gradual dying off of a certain number of cells, but this was not more noticeable with the experimental solution than with tap water for the

[4] Loss of chlorine accompanied by injury was not found to occur unless the pH value was below 4.8. The marked loss of chlorine and injury beginning at about pH 4.4 is correlated by Pearsall (Pearsall, W. H., and Ewing, J., *New Phytologist*, 1924, xxiii, 1923) with the isoelectric point of the *Nitella* proteins. With regard to the pH value of the cell sap, the various treatments were not found to alter appreciably the normal value of 5.2.

periods in question. In both types of media, under appropriate conditions of light and temperature, much new growth took place in the course of a month or more. After 2 or 3 months, cells kept in these solutions showed evidence of injury, but a complete culture solution

TABLE I.

Comparison of Concentration of Br and Cl in Sap from Cells Exposed to Different Types of Media Containing KBr or KCl.

Composition of medium.	Concentration of Br or Cl in cell sap.	Period of exposure.
	m.-Eq.	
Buffer solution + KBr 5.0 m.-Eq.	22.7 Br.	6 days continuous illumination.
KBr 5.0 m.-Eq., no buffer.	24.8 "	" " "
Buffer solution + KBr 5.0 m.-Eq.	57.0 "	17 days continuous illumination.
KBr 5.0 m.-Eq., no buffer.	49.5 "	" " "
Buffer solution + KCl 5.0 m.-Eq.	127.5 Cl.	6 days continuous illumination.
KCl 5.0 m.-Eq., no buffer.	118.0 "	" " "
Buffer solution + KCl 5.0 m.-Eq.	136.2 "	17 days continuous illumination.
KCl 5.0 m.-Eq., no buffer.	140.5 "	" " "
Buffer solution + KBr 5.0 m.-Eq.	20.5 Br.	6 days daylight.
Tap water + KBr 5.0 m.-Eq.	11.3 "	" " "

pH of phosphate buffer solutions + KBr and of KBr solutions 5.0–5.4. pH of tap water + KBr 7.0 or above.

Other data prove the lessened absorption from tap water was, to a large extent, caused by the influence of the chlorine ions.

(plus $CaCO_3$) containing bromide was as favorable a medium as tap water, judging by the new growth obtained during the course of a year.

Later studies showed that the accumulation[5] of Br was very similar

[5] For convenience of discussion, we are using the word "accumulates" in the sense proposed by Osterhout, *i.e.* when a substance reaches a higher concentration in the sap than in the surrounding solution.

whether KBr was used alone or added to the buffer solution (Table I). It may be noted that the former solutions were not completely free of traces of calcium, because of contamination from surface deposits on the cells. Therefore, no conclusion can be drawn with regard to the effect of a complete absence of calcium from the solution, but it is evident that in dilute solutions of this character, relatively high in K, the calcium added to the solution had no striking effect on the accumulation of Br, and that the use of buffer salts was not influential in determining the course of absorption.

Most of the experiments were carried out in beakers or wide mouth bottles with a capacity of 3 or 4 liters. From 100 to 125 gm. of cells, drained free of excess water, were placed in 3 liters of solution. The mass of cells was previously washed thoroughly in distilled water. At the end of an experiment the cells were removed from the solution, first washed with tap water and then with distilled water, after which the sap was recovered as already indicated. Except in experiments in which complete analyses were to be made, when several thousand cells were used, each sample of cell sap ordinarily represented several hundred cells and varied in volume between 2 and 4 cc. Because of the large number of cells which each sample of sap represented, errors resulting from the variability of individual cells were reduced sufficiently to permit satisfactory comparisons of different treatments. Incidentally, it may be remarked that while the whole procedure is exceedingly tedious and time-consuming, no easier way of obtaining direct evidence concerning the composition of the cell sap has been suggested, and it is just this type of evidence which is most needed at the present time.

Temperature Effects.

In this series of experiments, we desired to obtain some indication of the temperature coefficient for the absorption of Br under controlled light conditions. Two double walled baths were constructed with arrangements for flowing tap water or ice water. Heat was supplied by 100 watt lamps covered with tin-foil and immersed in the water contained in the inner compartment. The lamps were connected with a mercury thermoregulator capable of regulating the temperature of the inner bath to $\pm .1°C$. Mechanical stirrers were placed at one end of this bath.

The *Nitella* cells were contained in large beakers or jars set in the inner compartments and were illuminated by two 100 watt lights approximately 1 foot apart and suspended about 1 foot above the containers. Porcelain reflectors were used. It was found that the radiation from the lights caused the upper few inches of solution in

TABLE II.

Concentrations of Br in Sap from Nitella Cells after Exposure to Bromide Solutions Kept at Different Temperatures.

No. of experiment.	Temperature.	Concentration of Br in cell sap.	Temperature coefficient (10°C.).	Concentration of Br in expressed sap.	Temperature coefficient (10°C.).	Period of exposure.
	°C.	m.-EQ.		m.-EQ.		hrs.
1	10	.4	3.5	.3	2.7	6
	20	1.4		.8		
2	10	2.2	2.9	1.2	2.7	25
	20	6.4		3.2		
3	10	4.3	2.9	2.7	2.6	50
	20	12.3		7.0		
4	10	4.0	2.5	1.9	2.6	52
	20	10.0		5.0		
5	10	7.1	2.3	3.6	2.4	68
	20	16.6		8.7		
6	14	12.5	2.0	6.6	2.2	72
	24	24.4		14.7		

In Experiments 1, 5, and 6 the values are averages of duplicate experiments.

Phosphate buffer solutions + .005 M KBr. Initial pH 4.8–5.1. Final pH 5.6–5.8.

Continuous illumination with two 100 watt lights, suspended approximately 1 foot above the jars containing the cells. Temperatures of bath kept within ± .1°C., except for occasional short periods when adjustments were being made.

which the cells were immersed to be several tenths of a degree higher in temperature than the body of the solution, but the increase was the same for both temperatures compared. In any case, it was evident that the temperature control was much more accurate than the possible control of other factors. In this series of experiments, the illumination was continuous and daylight was excluded.

In Table II are presented results showing the concentrations of Br found in the sap of cells which had been exposed to solutions kept at temperatures 10°C. apart, *i.e.* 14–24°C., and 10–20°C. It would have been desirable to have made measurements at numerous intervals of time for each temperature, but this would have involved the use of many large containers kept under definite temperature control, for which no facilities were available. However, while velocity constants could not be calculated, the data, taken as a whole, seem to indicate quite clearly that the temperature coefficient (between 2.0 and 3.0 for 10°C.) for the absorption of bromine under the conditions specified is of the order of magnitude generally characteristic of chemical reactions rather than of diffusion processes, and this is the main point of interest at present.

Effect of Light on Accumulation of Br.

Earlier experiments showed that the removal of Cl from dilute solutions was definitely influenced by the conditions of illumination. Several preliminary experiments also indicated that the penetration of NO_3 or of Br into the sap of *Nitella* cells was likewise accelerated under the influence of light. In the first series of the present experiments, the temperature arrangements above described were utilized. The exclusion of light, when desired, was accomplished with large beakers painted black on the outside and covered loosely over the top with black paper. The periods during which the cells were exposed to the solution containing bromide (buffer solution plus 5 milli-equivalents KBr) were relatively short, but there was a marked difference between the illuminated and unilluminated cells in respect to the concentrations of Br present in the cell sap at the end of the experiment (Table III). Except in one instance, the concentrations of Br in the samples of sap obtained from the illuminated cells were from about two to four times those in the sap from the unilluminated cells.

Subsequently, numerous additional experiments were performed over longer periods of time, without the use of the temperature baths, but with such small differences in temperature between the illuminated and unilluminated cells as to be negligible for this purpose. In every case, exposure to light strikingly increased the ability of the cells to accumulate Br. In fact, when solutions of 5 milli-equivalents KBr

TABLE III.

*Concentration of Br in Sap from Illuminated and Unilluminated Nitella Cells after Exposure to Bromide Solutions.**

No. of experiment.	Temperature.	Concentration of Br in cell sap.	Light condition.	Period of exposure.
	°C.	m.-EQ.		hrs.
1	10	2.2	In light.	25
	10	1.7	" dark.	
	20	6.4	" light.	
	20	2.6	" dark.	
2	10	4.3	" light.	50
	10	1.9	" dark.	
	20	12.3	" light.	
	20	3.3	" dark.	
3	10	4.0	" light.	52
	10	2.2	" dark.	
	20	10.0	" light.	
	20	3.2	" dark.	

* Solutions, illumination, and temperature arrangements same as described in Table II.

TABLE IV.

Concentration of Br in Sap from Nitella Cells Exposed to Bromide Solutions in Continuous Darkness, with Longer Periods of Exposure.

No. of experiment.	Medium.	Period of exposure.	Concentration of Br in cell sap.
		days	m.-EQ.
1	Tap water and 5.0 m.-Eq. KBr, in dark.	5	5.0
	Same, in daylight.	5	11.2
2	KBr, 5.0 m.-Eq. in dark.	7	5.2
	Same, in daylight and artificial light.	7	19.3
3	KBr, 5.0 m.-Eq. in dark.	5	5.0
4	KBr, 5.0 " " "	2	2.5
5	KBr, 5.0 " " "	2	1.6
	Same, in dark.	6	4.3

KBr solutions without buffer salts.

These experiments were carried out at room temperature, averaging about 20°C. At the end of the longer periods in the dark, many small cells had died, but the larger cells from which sap was obtained were turgid.

were employed, the concentration of Br in the sap of cells kept in the dark did not exceed that present in the outside solution; while the sap from illuminated cells subjected to similar solutions for equal periods of time contained much higher concentrations of Br. This was true of several different media containing bromide (Table IV). When media with concentrations of 1 milli-equivalent KBr were employed, there was considerable evidence that the concentration of Br in the sap could exceed that of the solution, even under conditions of darkness, but the quantities involved were too small to admit of certainty.

It was not found possible to keep the cells in the dark for a very extended period because of injury which was accelerated by the development of microorganisms. The question then arose whether cells kept under a normal condition of alternating periods of light and darkness could concentrate Br in the sap during the periods of darkness. It appeared that this point could be tested by alternating solutions in such a manner that some cells would have access to Br only during the periods of illumination and other cells only during the periods of darkness. Accordingly, two experiments of this type were carried out. In the first experiment, the periods were divided approximately into 12 hours of illumination and 12 hours of darkness. At the end of each period, a transfer of solutions was made, after washing the cells very thoroughly with distilled water. The result of this experiment (Table V) was that the sap from the cells having access to Br only during the periods of illumination contained Br in a concentration several times that of the external solution, but in the sap from the cells immersed in the bromide solution only during periods of darkness, the concentration of Br was not greater than that of the outside solution.

This experiment did not indicate that there was any residual effect of light but it was deemed advisable to make a further experiment over a longer total period of time, with 24 hour periods of illumination followed by 24 hour periods of darkness. The solutions were changed in the same way as before. Again a marked difference was found between the cells kept under the two conditions, but the sap from the cells placed in the bromide solutions only during periods of darkness had accumulated Br in a concentration significantly greater than that

of the outside solution. The concentration in the sap was also much greater than could occur with cells kept an equal time in continuous darkness. Evidently, under suitable conditions, some effect of the

TABLE V.

Absorption of Br in Dark by Cells Exposed to Alternating Periods of Light and Darkness.

	Concentration Br in cell sap.	Concentration Cl in cell sap.
Experiment 1.		
(Approximately 12 hr. periods of alternating light and darkness. Total duration 101 hrs.*)		
	m.-Eq.	m.-Eq.
A. Cells having access to KBr (concentration 5.0 m.-Eq.) only when illuminated.	18.2	102.5
B. Cells having access to KBr (concentration 5.0 m.-Eq.) only when in dark.	3.9	116.7
Experiment 2.		
(Approximately 24 hr. periods of alternating light and darkness. Total duration 10 days.)		
A. Cells having access to KBr (concentration 5.0 m.-Eq.) only when illuminated.	37.8	96.4
B. Cells having access to KBr (concentration 5.0 m.-Eq.) only when in dark.	19.0	112.5
C. Cells having access to KBr (concentration 1.0 m.-Eq.) only when illuminated.	24.9	106.0
D. Cells having access to KBr (concentration 1.0 m.-Eq.) only when in dark.	6.4	115.8

Values for concentrations in sap, averages of duplicate experiments. Solutions were buffered with phosphate, as in other experiments.

Experiment 1.—Illumination by two 300 watt lights suspended about 1 foot above jars. Temperature 24°C. ± 1.0°C.

Experiment 2.—Illumination by two 300 watt lights suspended about 1 foot above jars. In this experiment, diffused daylight supplemented the artificial illumination. Temperature 20–25°C.

* First period in light was 17 hrs. duration.

illumination can be carried over to a subsequent period of darkness and influence the absorption or accumulation of substances. It cannot be stated at present whether or not this effect is concerned with a storage of available carbohydrates, although such a suggestion is a

natural one. We know that certain types of cells must always be confined to the indirect use of light energy through the oxidation of carbohydrates. In the case of the *Nitella* cells, the ability to store easily available sugars seems to be very limited.

The next experiment on the effect of illumination was planned for the purpose of determining how varying the periods of illumination during each 24 hours would influence the amounts of bromine concentrated in the cell sap. In this experiment, the jars containing the *Nitella* cells were placed in a glass chamber originally designed for studying the growth of wheat plants under controlled light conditions. The chamber was illuminated by six 500 watt Mazda lights (with

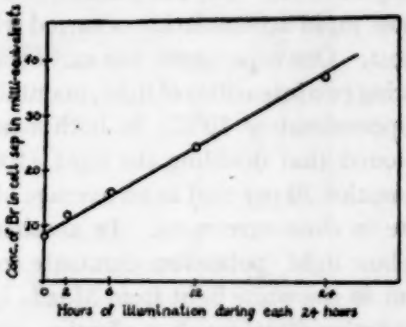

FIG. 1. Relation between number of hours of illumination and accumulation of Br in sap of *Nitella* cells. During periods of exposure, cells were illuminated by six 500 watt Mazda lights. (See text.)

metallic reflectors) arranged about the sides and ends of the chamber, and approximately 3 feet distant from the jars containing the *Nitella* cells. The temperature was controlled at 23–25°C. with the aid of an air current flowing through the chamber. The light was cut off from certain cells when required by placing over the jars cylinders of heavy brown paper with loose coverings for the top. This arrangement shut out practically, but not absolutely all the light. The illuminated solutions had a slightly higher temperature, about 1°C., than the others, but the effects of varying the periods of illumination were so great that this factor was negligible in comparison.

Referring to Fig. 1, it will be observed that there was a striking increase in the concentration of Br in the cell sap with each increased

period of illumination. The greatest concentration was found in the sap from cells exposed to light continuously for 3 days. The concentrations of Br do not vary in direct proportion to the periods of illumination, but an approximately straight line is formed by plotting the hours of light against Br concentrations. The total number of points is not sufficiently great to warrant an attempt at a mathematical analysis, but there can be no doubt of the influence of the length of the periods of illumination. This conclusion is in accord with our previous observations on the removal of Cl from dilute solutions.

With regard to the possible relations existing between quality or intensity of light and the accumulation of Br, it will be necessary to make additional experiments. For the present, it may be noted that, in general, the most rapid accumulation occurred where the intensity of light was greatest. One experiment was carried out for the specific purpose of comparing two intensities of light, maintaining the temperature the same (approximately 20°C.) in both cases within .2 of a degree. It was found that doubling the light (100 and 200 watts) increased the absorption 30 per cent as an average of duplicate experiments which were in close agreement. In another test, the results indicated that yellow light (potassium chromate solution filter), was at least as efficient as the white light from Mazda lamps. Blue light (alkaline copper solution filter) was less effective, but this observation has no necessary significance since the total energy values of the different lights are not known.

The Effect of Toxic Agents.

The ability of *Nitella* cells to concentrate various substances in their cell sap is apparently bound up with the processes of growth or metabolism. Toxic agents might, therefore, be expected to interfere with such a concentrating action. We have made several experiments which seem to support this assumption (Table VI). Different toxic substances were added to the bromide solutions and the concentration of Br in the cell sap determined as in the other experiments. In some cases, many small cells were killed by the toxic substances, but the samples of sap used for the analysis were, of course, obtained from those large cells which still remained turgid at the end of the period of exposure. It is reasonable to suppose, however, that these

cells also suffered some injury. Prolongation of the treatments would have resulted in the death of all the cells.

The injury has evidently caused, or been accompanied by, a decreased ability on the part of the cells to concentrate bromine in the

TABLE VI.

Effect of Toxic Agents on Concentration of Br in Cell Sap.

No. of experiment.	Treatment.	Concentration of Br.	Period of exposure.
1	None.	28.8	3 days continuous illumination.
	1 p.p.m. KCN.	29.0	
	10 " "	18.2	
2	None.	19.7	5 days daylight.
	Chloroform. (1 cc/3 l)	12.5	
3	None.	13.5	4 " "
	10 p.p.m. KCN.	14.1	
	20 " "	8.7	
4	None.	22.6	4 " "
	Chloroform. (1 cc/3 l.	9.4	
	Ether.	14.7	
	20 p.p.m. KCN.	12.5	
	Thymol excess.	12.5	

Phosphate buffer solutions containing 5.0 milli-equivalents KBr. 1 cc. of ether or chloroform added to 3 liters of solution. Treatment with ether repeated several times. Additional chloroform added once in Experiment 4. In Experiment 4, results are averages of duplicate experiments, except for last treatment. The differences between duplicates were much smaller than those between the control and the treated cells.

cell sap. Tröndle,[6] by indirect methods, has reached a somewhat similar conclusion with the use of various salts. While, in the case of anesthetics, the electrical resistance of certain plant cells may be increased over a limited period of time,[7] any marked injury generally involves increased permeability, as, for example, in the experiments

[6] Tröndle, A., *Biochem. Z.*, 1920, cxii, 259.

[7] Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Monographs on experimental biology, J. B. Lippincott Company, Philadelphia and London, 1922.

of Osterhout[8] and Brooks[9] on *Nitella* cells. In our view, such increased permeability may be accompanied by a decreased ability to concentrate substances in the sap. This important distinction will be referred to again in the later discussion.

The Exchange of Substances between Cell Sap and Culture Solution.

Before giving further consideration to the accumulation of Br in the cell sap, it is necessary to inquire into the possibility of an exchange of

TABLE VII.

Exchange of Br and Cl between Cell Sap and Culture Medium.

Composition of original solution.	Found in solution after contact with cells.		Period of exposure.
	Br	Cl	
Experiment 1.			
	m.-Eq.	m.-Eq.	*days*
Phosphate buffer + KBr 5.0 m.-Eq.....	3.85	.68	7
" " + KCl 5.0 "	.44*	3.66	
" " + KBr 5.0 "	2.44	1.38	43
" " + KCl 5.0 "	.90*	3.24	
Experiment 2.			
Phosphate buffer + KBr 5.0 m.-Eq.....	2.40	1.24	21

Cells kept at room temperature in daylight. The proportion of cells to solution was approximately 30 gm. of the former to 600 cc. of the latter.

The masses of cells were composed chiefly of small, very green and turgid cells, practically all in healthy condition, judged by appearance. No evidences of injury developed during the experiment. Similar cells kept in the phosphate solutions without Cl or Br did not lose these elements to the solution sufficiently to give a definite test with $AgNO_3$.

* The cells used in these tests had previously been exposed for several months to solutions containing .002 M KBr.

ions between the cell sap and the culture medium. Previous work had shown that the contents of the sap did not appear to diffuse out unless the cells were injured. When masses of healthy and uncon-

[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 709.

[9] Brooks, M. M., *J. Gen. Physiol.*, 1921–22, iv, 347.

taminated cells were immersed in a phosphate buffer solution, for example, the test for Cl in the solution was negative or only indicated a very slight trace, even after the lapse of several weeks. The same observation has been made with solutions containing sulfate or nitrate. But under exactly similar conditions, except with the substitution of bromide, very appreciable quantities of Cl were found in the external solution after from 1 to 6 weeks (Table VII), although in this time the cells presented no evidence of injury, judged by their appearance. In the course of a month, considerable new growth had occurred. The amounts of Cl lost from the cells were of such magnitude that they would have been followed by obvious signs of injury or death of the cells, had the loss occurred from cells exposed to solutions not containing bromide. Furthermore, when cells which had previously accumulated a high concentration of Br in their sap were placed in solutions containing chloride, Br entered the solution, but no appreciable amount was lost to similar solutions not containing chloride. For these reasons, it is very difficult to ascribe such exchanges to cell injury. If injury were present, it would seem that it must have been too slow to account for the effects noted. Another point to be emphasized is that Cl may be lost from cells at the same time Br is being concentrated in the sap. The studies on the Br and Cl content of the sap itself will now be described.

Accumulation of Br over Various Time Intervals.

In these experiments, Br determinations on the sap were made as before. Cl was determined on the same samples and the changes in its concentration were estimated by obtaining the difference between the concentration of Cl in the sap from untreated cells and in that from similar cells after exposure to the solutions under investigation. In many cases, the differences were comparatively large and since each sample was representative of a great many cells, the values for the increases or decreases of Cl concentration determined in this way are undoubtedly significant of loss or gain of Cl by the cell sap.

We now desire to direct attention to Fig. 2 in which are plotted the changes in Br and Cl concentrations of cell sap, which occurred over a period of 40 days. The cells were kept continuously illuminated and while the temperature could only be controlled very roughly, the

average temperatures for each time interval were sufficiently alike so that no important fluctuations in the general trend of the curves is noted. It will be observed, first, that the accumulation of Br takes place in a very gradual manner and that 40 days elapsed before a condition of apparent equilibrium was attained. This slow accumulation of Br in the cell sap was very definitely accompanied by slow loss of

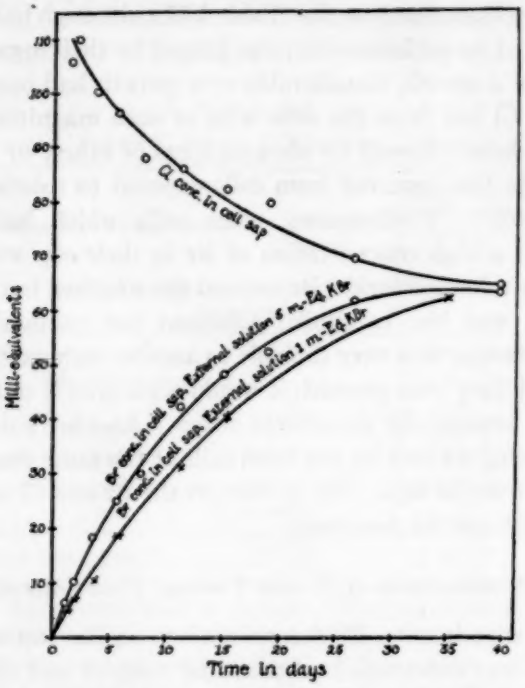

FIG. 2. Rate of accumulation of Br and loss of Cl over period of 40 days. Continuous illumination. Diffused daylight supplemented by artificial illumination by two 300 watt Mazda lights.

Values averages of duplicate or triplicate experiments, agreeing usually within 10 per cent. Error of 25 per cent may occur with very small concentrations of Br.

Cl until at the end of 40 days, the equivalents of Cl and Br present in the sap were almost equal. At this time, the masses of cells all appeared to be in a healthy condition.

While a loss of Cl took place, it is especially to be noted that the total halogen content of the sap was decidedly increased. In other

words, the equivalents of Br accumulated exceeded those of Cl lost from the cells.

In this experiment, comparison was also made of solutions containing 5 milli-equivalents and 1 milli-equivalent of KBr. The two curves representing the increases in the concentrations of Br in the cell sap are not widely separated and at the conclusion of the experiment, the concentrations in the sap of the cells kept in the solution of 1 milli-equivalent KBr and in that of 5 milli-equivalents KBr were almost the same. Evidently, there is no equality in the ratios of internal to external Br concentration with different concentrations of bromide present in the culture solutions. Further data pertaining to this point will be presented in a later discussion.

Recently Irwin[10] has subjected her data on the accumulation of a dye by *Nitella* cells to a mathematical analysis using the formula $K = \frac{1}{t} \log \frac{a}{a-x}$, in which K is a constant, t time, a concentration of dye in sap at equilibrium, and x concentration of dye at time t. It was thought that some interest might attach to the application of a similar formula to the data plotted in Fig. 2. (In this case, a and x refer to Br concentrations.) With the solution containing 5 milli-equivalents of KBr, a fairly good agreement is found between observed and calculated values of x, having in mind the difficulties of experimentation (Table VIII). The number of observations on the solution of 1 milli-equivalent KBr concentration was too small for the purpose but a lower value of K for this solution is indicated. With regard to the rate of loss of Cl, a lower value of K was obtained than for the accumulation of Br.

While the formula applied by Irwin to the accumulation of dye may fit approximately the data for the accumulation of Br, it does not, of course, follow that the two processes are necessarily similar. There is a very striking difference in the time required for the attainment of an equilibrium condition. In the case of the dye, this condition was reached in a few hours, while for Br 30 or 40 days was necessary, even under favorable light and temperature conditions. The ratio between the concentration of dye in the sap and in the external solu-

[10] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.

tion was found to be constant in the range studied, but such constancy of ratios is far from applying to the accumulation of Br. It was concluded that the dye very possibly combines with some organic constituent of the sap. There is no evidence that the accumulation of Br can be explained on the basis of organic combinations in the vacuole, although it may well be true that intermediate processes involve such combinations with some constituent of the protoplasm.

TABLE VIII.

Accumulation of Br in Cell Sap of Nitella.
(Fig. 2.)

Calculated according to formula used by Irwin for accumulation of dyes.

$$K = \frac{1}{t} \log \frac{a}{a - x}$$

k = constant, a, concentration of Br at equilibrium.
x = concentration of Br at time t.
a = 64.6.
Br concentrations in milli-equivalents.

Time.	K	x observed.	x calculated. $K = .037$
days			
1.15	.036	6.1	6.3
2	.037	10.1	10.1
4	.037	18.6	18.7
6	.037	25.8	25.8
8	.034	30.4	31.9
12	.039	42.8	41.4
16	.038	48.4	48.1
20	.038	53.4	52.9
28	.038	63.2	58.7
40	.036	64.6	62.5
Average.....	.037		

Culture solution phosphate buffer + KBr, 5 milli-equivalents, same as used in other experiments.

Other Reciprocal Relations of Br and Cl.

In connection with the displacement of Cl by Br, the question arose whether cells with Cl content initially higher than that of normal sap

could accumulate Br with the same rapidity as normal cells (*i.e.* cells kept in tap water). Several experiments were made with this point in mind. During an initial period of 10 to 14 days, certain lots of cells were exposed to a buffer solution without Cl and others to a similar solution to which 5 milli-equivalents of KCl had been added. At the end of this initial period, the cells (A) which had been in the solution without Cl were transferred to a solution containing 5 milli-equivalents KBr. A portion of the cells (B) which had been kept in the chloride solution were also placed in a solution containing KBr. Another portion of the cells (C) from the chloride solution was transferred to a solution without Cl or Br. The second period of the experiment was from 5 to 9 days in length. In each experiment cells were maintained under the same light and temperature conditions. At the end of the final periods, samples of sap were obtained from the various lots of cells and determinations of Cl and Br were made (Table IX).

Attention is first called to the fact that the Cl content of the cell sap (C) could be increased from 30 to 40 per cent under the conditions described. These and other experiments suggest, however, that the accumulation of additional Cl is less rapid than the accumulation of Br. The second important observation is that the cells (B) which had previously had their Cl concentration increased accumulated a much smaller amount of Br than did the cells (A) which had a normal Cl content in the sap at the time they were placed in the bromide solution. In the latter cells, a considerable concentration of Br was attained without anything like an equivalent displacement of Cl, but the accumulation of Br by the cells with the initially higher Cl concentration was at the expense of a nearly equivalent displacement of Cl. The cells behaved as though the total halogen content could be increased to a certain point, after which accumulation of halogens occurred only as a result of exchange or displacement. The consistent data obtained in three independent experiments under different conditions, seem to warrant these statements.

Conductivity Data.

If the total electrolyte content of the cell sap can be increased, we should be able to show that increases in conductivity also occur. In order to determine the extent of such changes, a special conductivity

TABLE IX.

Retarding Effect of Preliminary Accumulation of Cl on Subsequent Accumulation of Br.

Culture solutions (a).	Concentration of Cl in cell sap.	Concentration of Br in cell sap.	Light and temperature conditions.
	Experiment 1.		
	m.-EQ.	m.-EQ.	
A. Period 1, no Cl or Br added. " 2, KBr, 5 m.-Eq.	97.2	32.0	Period 1, 14 days; Period 2, 9 days. Continuous illumination (b).
B. " 1, KCl, 5 " " 2, KBr, 5 "	122.0	13.8	Same conditions.
C. " 1, KCl, 5 " " 2, no Cl or Br added.	137.5	0	" "
	Experiment 2.		
A. Period 1, no Cl or Br added. " 2, KBr, 5 m.-Eq.	103.0	40.1	Period 1, 10 days; Period 2, 5 days. Continuous illumination (c).
B. " 1, KCl, 5 " " 2, KBr, 5 "	117.5	22.7	Same conditions.
C. " 1, KCl, 5 " " 2, no Cl or Br added.	141.0	0	" "
	Experiment 3.		
A. Period 1, no Cl or Br added. " 2, KBr, 5 m.-Eq.	101.5	22.4	Period 1, 10 days; Period 2, 6 days. Continuous illumination (d).
B. " 1, KCl, 5 " " 2, KBr, 5 "	129.5	10.9	Same conditions.
C. " 1, KCl, 5 " " 2, no Cl or Br added.	137.2	0	" "

All experiments carried out in duplicate, with agreement within 10 per cent of total value, except in one case. Average values given.

(a) Phosphate buffer solutions + 5 milli-equivalents KBr or KCl, as indicated. pH approximately 5.4

(b) Illumination by two 300 watt lamps placed about 2 feet above jars, in addition to diffused daylight. Temperature average 20–22°C.

(c) 3 days illumination as in b, remainder of first period and all of second period illumination by six 500 watt lamps in light chamber. Temperature average, 18–20°C.

(d) First period in light chamber as in c; second period illumination as in b.

The Cl content of the untreated cells was as follows:

Experiment 1–101 milli-equivalents.
" 2–103 "
" 3–108 "

TABLE X.

Comparison of Conductivities of Normal Cell Sap and Sap from Cells Exposed to Solutions Containing KBr.

(Phosphate buffer solutions with 5 milli-equivalents KBr.)

Collection of cells.	Conditions of exposure.	Concentration of Cl in cell sap.	Concentration of Br in cell sap.	Specific resistance of cell sap.	Percentage decrease in resistance.
		m.-EQ.	m.-EQ.	*ohms*	
B	6 hrs. continuous illumination (1).	98.6	1.4	74.7	
B	6 days, same conditions (1).	95.4	37.8	59.4	21
B	9 " " " (1). (KBr, 20 m.-Eq.)	97.0	53.1	56.2	25
C	No treatment.	105.5	0	77.2	
C	10 days (7 days intermittent and 3 days continuous illumination).	90.9	49.6	62.3	19
C	17 days daylight (3).	83.4	33.4	69.2	10
C	38 " continuous illumination (4).	69.1	64.8	58.9	24
D	No treatment.	101.1	0	74.3	
D	10 days (4 days daylight and 6 days continuous illumination) (5).	Not determined.	64.5	51.2	31
D	9 days continuous illumination (6).	98.0	31.4	59.0	21
D	19 days daylight (7).	88.0	51.4	59.6	20
E	No treatment.	103.0	0	76.4	
E	16 days continuous illumination (8).	82.2	48.4	61.8	19
E	18 days continuous illumination (9).	80.0	56.6	54.8	28
E	20 days continuous illumination (10).	81.0	53.4	60.1	21
E	5 days continuous illumination (11).	105.0	41.0	54.9	28

(1) Continuous illumination by two 300 watt lamps, suspended about 1 foot above jars. Temperature 22–26.5°C.

(2) 7 days, diffused daylight, supplemented by two 300 watt lamps during periods of 7–8 hrs.; 3 days light on at night also. Temperature 20–25°C.

(3) Diffused daylight only. Temperature average approximately, 20°C.

(4) Diffused daylight in addition to continuous illumination with two 300 watt lamps, 2 feet above jars. Average temperature 20–22°C.

(5) Diffused daylight and illumination during 6 nights by two 300 watt lamps, 1 foot above jars.

(6) Same conditions as in (4).

(7) Diffused daylight only. Room temperature.

(8) Same conditions as in (4).

(9) Illumination by six 500 watt lights, light chamber. Average temperature approximately 20°C.

(10) Same conditions as in (4).

(11) " " " " (9).

cell was constructed for use with slightly less than 1 cc. of cell sap. Conductivity determinations were made in various experiments and certain typical data are given in Table X. In every case in which Br accumulated to any great extent a significant increase of conductivity occurred. These increases varied from 10 to 31 per cent, based on the values for sap from untreated cells. In certain instances, the increases of conductivity were of a magnitude very similar to those which would be obtained by adding to normal sap an equivalent amount of KBr, but in other cases, the increase is decidedly less than would correspond to this condition, as might be expected, considering that Cl ions have been displaced. Unpublished data indicate that an exchange of bases, especially sodium for potassium, may also occur under some circumstances. If these exchanges are taken into consideration, there is nothing in the conductivity data to suggest that an appreciable amount of Br has been organically combined. Other reasons for this view, previously discussed in connection with the chloride content of the sap also apply to the bromide content.

While the quantitative responses of different lots of cells collected at different times of the year are not exactly the same, there are one or two general relations which are strongly suggested by the available data. In several experiments, a large accumulation of Br took place with little or no loss of Cl. In these cases, the light and temperature conditions were very favorable to the accumulation and a high concentration of Br was attained in the cell sap in a relatively short period of time. In comparing two specific experiments conducted under different conditions, we note that in 6 days, with a high intensity of continuous illumination, 37.8 milli-equivalents of Br were accumulated with a loss of 3.2 milli-equivalents of Cl. In the other experiment, with diffused daylight alone, and with a lower temperature, 17 days were required to accumulate 33.4 milli-equivalents of Br and the loss of Cl was 22.1 milli-equivalents. While these relations can only be suggested at the present time, it is evident at least that a gain of Br and loss of Cl do not proceed necessarily at the same rate and that light, temperature, and time are important factors in all processes.

It cannot be proved, of course, that exchanges of ions are, strictly speaking, involved. Since it is also possible that cations may be

displaced, as well as anions, it could be assumed, for example, that KBr or K and Br ions entered the cell and that NaCl or Na and Cl ions left the cell. However, the course of events is certainly more complex than this statement would imply.

There are various other data which can be discussed later when certain additional experiments have been made, but a few preliminary observations may be made now incidentally. With regard to the effects of hydrogen ion concentration on the accumulation of Br, our present results do not show that these effects are the same as those obtained in the studies on nitrate, which accumulates much more slowly than Br. The influence of certain other anions on the accumulation of Br is striking, and in single salt solutions, the nature of the cation plays a very important rôle.

DISCUSSION.

In most of the investigations on the absorption of substances by plant cells, the latter are assigned a more or less passive rôle and the principal conclusions are concerned with explanations of alterations of permeability occurring under diverse conditions. This, however, can be only a partial view of the situation. From the point of view of the growth and nutrition of the plant, it is highly essential to emphasize the ability of the cell to concentrate, or, in the sense of the word as recently employed by Osterhout, to accumulate substances in its interior. All the evidence now available shows that it is possible for certain inorganic elements to be taken out of a dilute solution and stored in a solution of much higher concentration inside the cell. It is true that such processes may take place relatively slowly, but, nevertheless, often at an appreciable rate, as we have shown with regard to Cl and Br. In most physiological experiments which have been reported, no adequate idea of the intake of inorganic elements by plant cells has been obtained because the time intervals employed were too short.

The ability of living cells to concentrate substances has, of course, been recognized by various writers and in this connection, it seems worth while to quote the following from a recent book by Lillie:[11]

[11] Lillie, R. S., Protoplasmic action and nervous action, University of Chicago Press, Chicago, 1923.

"But since these compounds (crystalloidal compounds) do, in fact, gain entrance to the cell, at least at certain times, it is clear that the problem of cell permeability is not a simple one. Apparently, we must conclude that the entrance or exit of substances by simple diffusion is, in most cases, a different phenomenon from their entrance, or exit, under physiological conditions. The processes of absorption and secretion are, in fact, special activities, requiring the performance of work by the cell. The distinction between a passive or purely physical permeability and an active or physiological permeability thus seems to be a necessary one."

In view of the importance of such distinctions, it is regrettable that the term permeability has been used to describe so many types of phenomena. The general adoption of the term "accumulation" to designate certain processes, as in the recent usage of Osterhout, might serve to clarify discussion.

The experimental data presented in this article seem to offer definite evidence that light is an essential factor in the accumulation of Br or Cl by *Nitella* cells. Blackman and Paine, Tröndle, Lepeschkin[12] and several others, have described the effects of light on permeability, but in these investigations, the methods were indirect and permeability, as such, was the chief point of interest. Results such as we have obtained cannot be explained by the statement that light simply increases cell permeability. Light obviously contributes energy to a system, and it would seem necessary to assume that this energy, which, under appropriate conditions can be stored, may be utilized to bring about a movement of solutes from a region of low concentration to one of higher concentration.

In our earlier work, which has been confirmed, we found that Cl did not diffuse out of uninjured cells into the various types of solution which were tried, and it was questioned whether such outward movement of Cl could take place in the absence of injury. It now seems certain that an exchange of Br for Cl may take place (or its equivalent), and present information would not point to injury as the primary cause of the exchange, which seems to occur to an appreciable extent only with elements which can be absorbed or accumulated by the cell with relative rapidity. Notwithstanding the existence of such exchange phenomena, Br and Cl can both accumulate in the cell in

[12] See review by Stiles, W., Permeability, Wheldon and Wesley, London, 1924.

concentrations much higher than those present in the external solution, so that our views with regard to concentration gradients are not altered, but only extended.

It is now of interest to consider the suggestions recently advanced by Osterhout[13,14] to the effect that ions may not be able to penetrate living protoplasm, but that penetration is confined to undissociated molecules. We have been accustomed to assuming, in dealing with solutions of strong electrolytes such as those used in the experiments on *Nitella*, that the interpretation of the results could best be made in terms of ions. The dilute character of the solutions, existence of exchange phenomena apparently involving ions, the influence of one ion on the absorption of another, are some of the reasons which would seem to favor an interpretation in terms of ions, as far as the particular phenomena we have been investigating are concerned.

It is very possible the experiments carried out by Osterhout on certain chemical systems are not inconsistent with an hypothesis of ionic penetration as supplied to other systems. In his studies on the absorption of H_2S and CO_2 by *Valonia*, evidence was found that the equilibria between the sap and the sea water medium could be explained most logically on the basis that ions could not penetrate the cell. The question then arises whether the failure of the ions of the H_2S and CO_2 systems to penetrate necessarily implied that Br and Cl ions cannot do so. While the question is not now capable of a definite answer, several points of difference between the experiments with H_2S and CO_2, and the experiments with Br and Cl should be noted. In the first place, the time periods involved are very much longer in the latter case, so that opportunity would be afforded for the very slow entrance of ions. In the case of the studies on H_2S, with *Valonia* it was not found that a higher concentration of sulfide was attained inside the cell than outside, while *Nitella* cells show a marked ability to concentrate Br or Cl in the sap. Then, in the H_2S and CO_2 systems, gaseous components were present, and it is quite possible that these were involved in such a way as to make it very uncertain whether any direct comparison can be made between the results

[13] Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.
[14] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131.

obtained with Cl and Br, and those with CO_2 and H_2S. Still another point of difference is found in the temperature coefficient. Osterhout found a low coefficient for the entrance of H_2S, while in our experiments the coefficient for the accumulation of Br is of the order of magnitude of a chemical process.

Looking at the whole question from another point of view, it is not certain that we are now in a position to decide whether we are dealing with undissociated molecules or with ions, for the reason that intermediate chemical compounds may be formed, the chemical nature of which is unknown. If combinations of this sort are, in fact, involved, information concerning their character and the energy relations of their formation or dissociation would appear to be indispensable to an understanding of the mechanism of penetration of those substances which can accumulate in the cell sap of plants. It will also be necessary to determine whether these phenomena are concerned at all with electrical potential differences. In any case, the energy relations implied in the ability of living plant cells to concentrate substances require consideration irrespective of the mechanism of accumulation.

CONCLUSIONS.

1. By the use of a special analytical technique it has been possible to study the accumulation of halogens in the cell sap of *Nitella*.

2. From a dilute solution, Br may be accumulated in the sap in a concentration much greater than that of the external solution. The conductivity of the sap may be markedly increased by such accumulation. The process is a slow one so that a month or more may be required to approach equilibrium.

3. Cl may be lost from the cell as a result of the accumulation of Br and *vice versa*. Other reciprocal relations between Cl and Br are indicated.

4. At equilibrium practically as much Br accumulated in the sap with an external solution containing 1 milli-equivalent of Br as with one containing 5 milli-equivalents.

5. Light energy was indispensable to the accumulation of Br. The temperature coefficient was characteristic of a chemical process.

THE EFFECT OF THE pH ON THE GERMICIDAL ACTION OF SOAPS.

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That soaps possess a marked germicidal action has been known since the time of Robert Koch (1881). This valuable property finds every-day application in the sterilization of the skin, dishes, and soiled clothing. Soaps are furthermore of interest to the bacteriologist because they, or substances similar to them, very probably have something to do with the body's defence against infections (see Flexner's introduction to the paper of Lamar, 1911, *a*).

Solutions of soaps having 12 or more carbon atoms in the molecule are alkaline in reaction, because of hydrolysis. The longer the chain of carbon atoms, the greater is the hydrolysis and the more alkaline is the solution. Most investigators of the germicidal action of soaps are agreed that this alkaline reaction favors the destructive effect on the bacteria. Reichenbach (1908), working with *B. coli* as test organism, found that the more strongly hydrolyzed and therefore more alkaline soaps were most germicidal; addition of alkali increased the germicidal titer. Lamar (1911, *b*) found sodium oleate to be more hemolytic than oleic acid; this difference he ascribed to the lower solubility of the acid. Nichols (1919–20) found that the soaps commonly used in washing dishes (the resinates, stearates, and palmitates) were precipitated and lost their germicidal action when the reaction was brought to pH 7.0 or more acid. Further than this, there seem to be no observations recorded in the literature on the effect of the pH on the germicidal action of the soaps.

EXPERIMENTAL.

Soaps of the following normal fatty acids were investigated: butyric, caproic, caprylic, capric, undecylic, lauric, tridecylic, myristic,

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pentadecylic, palmitic, stearic, oleic, and ricinoleic. The butyric, caproic, and oleic acids were Kahlbaum's; the remainder were obtained from the Eastman Kodak Company. The soaps were prepared by adding the theoretical quantity of fatty acid to N/5 KOH. Potassium soaps were chosen because of their greater solubility. Walker (1924) has shown that the sodium and potassium soaps have prac-

TABLE I.

Composition of Buffer Mixtures.

pH	M/5 KH_2PO_4	N/5 KOH	N/10 glycine.	N/10 KOH
	cc.	cc.	cc.	cc.
5.0	9.90	0.10	10.0	0
5.5	9.71	0.29	10.0	0
6.0	8.97	1.03	10.0	0
6.5	7.67	2.33	10.0	0
7.0	6.28	3.72	10.0	0
7.5	5.50	4.50	10.0	0
8.0	5.17	4.83	9.75	0.25
8.5	5.10	4.90	9.0	1.0
9.0	5.10	4.90	8.0	2.0
9.5	5.10	4.90	6.5	3.5
10.0	5.10	4.90	5.5	4.5

TABLE II.

Composition of Lactic Acid-Potassium Lactate Buffer Mixtures.

pH	N/10 potassium lactate.	N/1 lactic acid.
	cc.	cc.
5.0	10.0	0.0625
4.7	10.0	0.125
4.4	10.0	0.25
4.1	10.0	0.50
3.8	10.0	1.00

tically the same germicidal value. Before each experiment, serial dilutions of the soaps required were made with sterile distilled water.

The pH of the soap solutions was controlled by the addition of buffer mixtures, whose composition is given in Tables I and II.

The composition of the phosphate and glycine buffers was calculated from the data given in Clark (1922). The pH of the glycine-

KOH mixtures is for 37°C. The phosphate mixtures were made up in larger quantities; the glycine-KOH mixtures were made up fresh for each experiment and added to the phosphate.

Five different species of test organisms were used. These were:

1. *Streptococcus pyogenes.* The strain used was the "H" strain first described by Gay and Stone (1920); its extremely high virulence for rabbits has been maintained by constant animal passage. This strain is hemolytic; it grows vigorously and diffusely in infusion broth.

2. *B. diphtheriæ.* The "Park-Williams No. 8" was the strain used.

3. *Staphylococcus aureus.* An old laboratory stock culture was employed.

4. *B. typhosus.* The "Pfeiffer" strain was used.

5. *Vibrio choleræ.* The strain used was an old stock culture of unknown origin.

The germicidal tests were performed in the following manner:

The required buffer solutions were sterilized; when cool, they were inoculated with the test organisms and well mixed. Then 0.5 cc. quantities were pipetted into series of small sterile test-tubes. The inocula were such that each 0.5 cc. of the buffer fluid contained 0.04 cc. of an 18 to 24 hour broth culture of *Streptococcus pyogenes, B. diphtheriæ,* or *V. choleræ;* or 0.02 cc. of *Staphylococcus aureus* or *B. typhosus.* Then 0.5 cc. quantities of the soap solutions (serial dilutions of which were prepared in advance) were added to each tube. Finally each tube was gently rotated while in a slanting position and placed in the water bath at 37°C. At the end of 30 minutes, 2 hours, and 18 hours, a 4 mm. loopful from each tube was subcultured on plates of blood agar (*Streptococcus, B. diphtheriæ,* and *V. choleræ*) or plain agar (*Staphylococcus* and *B. typhosus*). In case the tube contained a precipitate of insoluble fatty acid, the precipitate was well stirred with the platinum loop on subculture. The plates were incubated at 37°C. for 48 hours and read. In several experiments duplicate subcultures were made: the first set on agar plates, the second set in tubes of infusion broth. No essential differences were observed.

The soap solutions added were in certain instances strongly alka-

line. The question thus arises, how adequate are these buffer mixtures to resist change of pH on the addition of this alkali? The alkaline end of the series—from pH 8.0 on—is very well buffered against weak alkalies such as the soaps; the acid end, on the other hand, is but poorly buffered against any but very dilute solutions. It was found necessary to test the final pH of the solutions with indicators to determine where any serious shifting of pH had occurred. The less hydrolyzed soaps gave very little trouble. Those with fewer than 10 carbon atoms could easily be adjusted with acid before being added to the buffer mixtures. The potassium laurate, in concentrations

TABLE III.

	Acid limit of tolerance.		Alkaline limit of tolerance.	
	2 hrs.	18 hrs.	2 hrs.	18 hrs.
	*p*H	*p*H	*p*H	*p*H
Streptococcus	5.5	6.0	9.5	9.0
B. diphtheriæ	4.4	4.7	10.5	10.0
V. choleræ	5.5	6.0	9.0	8.5
Staphylococcus	3.8	4.4	10.5	10.0
B. typhosus	4.4	5.0	10.0	9.0

The pH values given indicate the most acid or alkaline tube that gave growth on subculture. The tests were conducted in the same manner as the germicidal tests with soap, except that distilled water instead of soap solution was added to the buffer fluids.

of N/160 or less, caused no serious shifting of pH except in the most acid of the phosphate mixtures. The same was true of the soaps of higher molecular weight when the concentration was N/320 or less. When any shift in the pH of a significant tube occurred, the observed pH (colorimetric) was recorded. As a matter of fact, corrections of this nature were not often required.

Inasmuch as an excess of either acid or alkali is destructive to bacteria, repeated tests were made to determine the acid and alkaline limits of pH tolerated by the five organisms here studied. The results are given in Table III.

The results of the germicidal experiments with soaps are given in Figs. 1 to 7. Attention must be called to the fact that the ger-

micidal titers given in these figures refer, not to the amount of soap or fatty acid in solution, but to the total amount added, irrespective of whether a precipitate formed or not Thus, when N/10 potassium stearate was brought to pH 6.0, practically all of it was precipitated out. When such a mixture was found to be without germicidal properties, it was recorded as having a titer of <N/10. Again, when N/640 potassium myristate was brought to pH 6.0 (as in Fig. 1) a precipitate formed, and the actual concentration of myristate in solution became much less, yet it was recorded as N/640. This procedure was adopted for two reasons: first the difficulty of estimating the amount of fatty acid actually in solution; and second, the fact that the substances in solution are in equilibrium with the precipitate, so that the latter cannot be regarded as wholly inert.

A study of the experimental data brings out the following facts.

1. Potassium butyrate is non-germicidal in a concentration of N/10 at all pH values within the limits given in Table III.

2. The lower members of the saturated series of soaps are most germicidal in an acid reaction. The most striking example of this was found in the case of potassium caprate and *Staphylococcus:* the titer here was 1000 times as great at pH 4.4 to 4.7 as at pH 9.0 to 10.0.

3. The curves for the higher members of the saturated series are the reverse of those for the lower members, showing greater germicidal action when the pH is alkaline. Certain soaps (such as the myristate and the palmitate with *Streptococcus*) are intermediate in their behavior, showing a definite minimum in their activity near the neutral point.

4. With increasing molecular weight of the soap, the germicidal titer increases to a maximum and then diminishes. The point at which this inflection occurs varies with the pH and the organisms (Figs. 5 and 6). Thus, with *B. typhosus* at pH 5.5, the titer rises from caproic to capric acid, and then falls off very rapidly through undecylic acid to lauric acid, where no germicidal action could be demonstrated. With the other four organisms tested, the maximum for the acid range was reached at lauric and tridecylic acids, with a somewhat slower falling off of the titer with increasing molecular weight. In the alkaline pH range, the germicidal action toward all five organ-

isms increases with the molecular weight to the palmitate and then diminishes.

5. The oleate and ricinoleate were not tested against all five organisms. Fig. 7 shows that the oleate with *Streptococcus* and the ricinoleate with *B. diphtheriæ* are far more effective in acid than in neutral or alkaline ranges. The oleate with *B. diphtheriæ* gave a curve quite unlike any other that was obtained, with maximal germicidal action at pH 6.5 and 7.0. The oleate showed no action on *Staphylococcus* and very little on *B. typhosus*, and then only at the most alkaline reactions. This confirms the observations of Reichenbach (1908) and Walker (1924).

From this data it is clear that the soaps have a germicidal power that varies with their structure and the species of germ acted upon; and that the corresponding fatty acid may likewise be germicidal, often far more than the soap.

The question arises, what is the cause of the sudden drop in the curves (Figs. 5 and 6) after a maximum has been reached? Why are the curves in Figs. 1 to 4 reversed as we pass from soaps of lower to higher molecular weight?

One reason for this is the diminishing solubility of the soap or fatty acid as the molecular weight increases. Thus, the most concentrated solution of potassium laurate that remains clear at pH 6.0 is N/5120. More concentrated solutions contain insoluble matter in suspension. It is impossible to determine whether N/160 dissolved laurate or palmitate would kill the typhoid bacillus at pH 6.0, because that amount cannot be obtained in solution.

There is also evidence to show that the germicidal power of the fatty acids also actually diminishes with increasing molecular weight, after a maximum has been reached. It is suggested that this may be due to diminished solubility of the germicide in the bacterial protoplasm. In the case of *B. typhosus* (Fig. 6), the sudden drop from capric to undecylic acid can be explained only by a diminution in germicidal power.

A comparison of the germicidal titers for the three different time intervals gave some rather interesting results. The following combinations ran practically the same titers for the 30 minute, 2 hour, and 18 hour periods: *Staphylococcus* with soaps of 12 or fewer carbon

atoms; *B. typhosus*, *B. diphtheriæ*, and *V. choleræ* with 11 or fewer carbon atoms. With soaps of higher molecular weight, the titers were increased by lengthening the incubation period. This increase, however, was not always uniform over the whole pH range. Thus, for the 18 hour period, the curve for the myristate and *Staphylococcus* (Fig. 1) was flattened out without being raised at the ends, while the palmitate and stearate curves were shifted to the left without any change in their shape. On the other hand, the myristate curve for *V. choleræ* (Fig. 3) instead of being flattened, was strongly raised at both ends when the incubation period was increased to 18 hours.

The tests described were carried out in a salt concentration of N/20. A detailed study of the effect of salt on the germicidal action of soaps

TABLE IV.

The Effect of the Salt Concentration on the Germicidal Titer of Potassium Laurate on B. diphtheriæ.

Salt concentration. (Potassium phosphate.)	Germicidal titers.		
	pH 6.0	pH 7.0	pH 8.0
N/10	N/10,240	N/1,280	N/320
N/25	N/5,120	N/640	N/160
N/100	N/2,560	N/320	N/160

Temperature, 37°C. Time of incubation, 2 hours.

was not undertaken, but a few experiments were made which indicated that the addition of salts increases the germicidal action. One such experiment is shown in Table IV.

This effect of salt upon the germicidal action of soaps and fatty acids is similar to the effect of salt upon phenol, another organic acid. In the latter case, the effect of salt was explained by Spiro and Bruns (1898) in this manner: When bacteria are added to a solution containing phenol, the equilibrium concentrations of $\frac{\text{phenol in bacteria}}{\text{phenol in water}}$ will depend upon the relative solubility of the phenol in the two phases. If anything is added to the water phase (such as alcohol) that increases the solubility of the phenol in it, then the concentration of phenol in the bacteria is diminished, and likewise germicidal action. Con-

versely, as salt diminishes the solubility of phenol in water, it increases the phenol concentration in the bacteria, causing increased germicidal action. As salts diminish the solubility of soaps and fatty acids in water, the reasoning of Spiro and Bruns would equally well explain the salt effect here. It should be noted, however, that where the added salt actually precipitates the soap out of solution such enhancement of germicidal action is not to be expected.

If we consider the germicidal titer of soaps for *Streptococcus*, we see that with only one exception (potassium stearate) the different soaps tried were more active in acid than in neutral or alkaline solutions (Figs. 2 and 7). It seems probable that the stearate failed to show this phenomenon because its acid is too insoluble. With *B. diphtheriæ*, *Staphylococcus*, and *V. choleræ*, the palmitate, pentadecylate, and myristate likewise fail to be effective in acid ranges; and with *B. typhosus*, which is the most resistant organism of the group, the tridecylate, laurate, and undecylate are added to the above. In each case it seems likely that the germicidal activity of the fatty acid fails to become manifest because it is not soluble in the necessary concentration. Certainly, in all cases where the acid is sufficiently soluble to show its true germicidal action it is more active than the corresponding soap. In this there is again an analogy with phenol, as Scheurlen and Spiro (1897) showed that phenol is a more powerful germicide than sodium phenolate.

Several factors suggest themselves as being involved in this increased activity in acid solutions:

1. The primary effect of the pH may be on the bacterium rather than on the germicide; an acid reaction may favor germicidal action by rendering the bacterium more sensitive to the soap or fatty acid.

2. An acid reaction may diminish the surface tension of the soap solution, and hence greatly increase the concentration of the germicide at the surface of the bacterium. For soaps containing 12 or fewer carbon atoms this is actually the case. For soaps containing more than 12 carbon atoms the reverse is true (Jarisch, 1922–23). The extreme insolubility of the latter in acid buffer fluids, however, makes surface tension measurements rather unsatisfactory.

3. The effect of the pH may be due to alterations of the solubility of the soaps in the aqueous phase or in the bacterial protoplasm.

This would affect the distribution coefficient of the germicide between germs and test fluid. We do not know what changes occur in the pH of living bacterial protoplasm when the pH of the outside fluid is altered. However, Coulter (1924–25) has shown that when acid is added to a suspension of erythrocytes, the cell contents do not become as acid as the outside fluid. If this is the case with bacteria, an acid outside pH would be accompanied by a much less acid pH of the bacterial protoplasm. As acids diminish the solubility of soaps, an acid reaction of the outside fluid should, therefore, increase the concentration of soap (or fatty acid) in the bacteria. If, however, the added acid precipitates most of the soap out of solution, the actual concentration within the bacteria would be diminished, even though the relative concentration is still greater than that in solution in the outside fluid.

4. The greater germicidal activity of the fatty acid may be due to the fact that the acid is less dissociated than the soap. There is a good deal of evidence to show that undissociated molecules penetrate more readily into protoplasm than do ions (Osterhout, 1925–26). Michaelis and Dernby (1922) believe that the germicidal effect of the organic bases studied by them is due entirely to the undissociated molecules; they show that the curve for the germicidal titer closely parallels the dissociation residue curve for a weak base. Scudder (1914) gives the value of K_a for caprylic acid as 1.44×10^{-5}; the dissociation residue for this acid corresponds very closely with the germicidal titer curves for the same acid (Figs. 1 to 4). The latter, however, are logarithmic curves; hence it would appear that the logarithm of the germicidal titer varies with the dissociation residue of this acid. This means either that other variables enter into the germicidal effect, or that the dissociation residue affects the germicidal titer in two or more different ways (*e.g.* by affecting the surface tension or the distribution coefficient as well as the ability to penetrate).

SUMMARY.

1. The effect of the pH on the germicidal action of soaps has been studied. The lower members of the series were found to be most active in acid solution; the higher members, in alkaline. The point of transition varied with the test organism used.

2. This is probably due to the effect of the pH on the dissociation residue and on the solubility of the soap. The dissociation residue may affect the germicidal titer by modifying the surface tension, the penetration into the bacteria, and the partition coefficient of the germicide between bacteria and water.

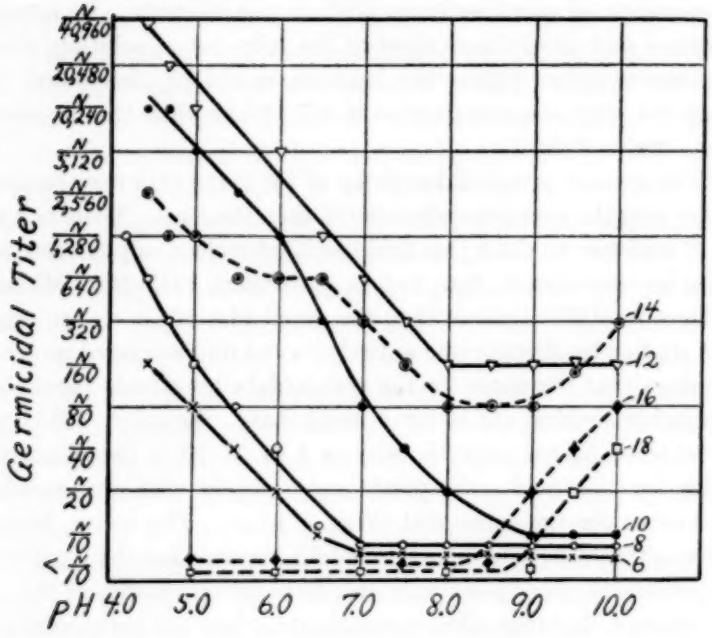

FIG. 1. Germicidal titers of saturated soaps for *Staphylococcus aureus*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted from the figure for simplification; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

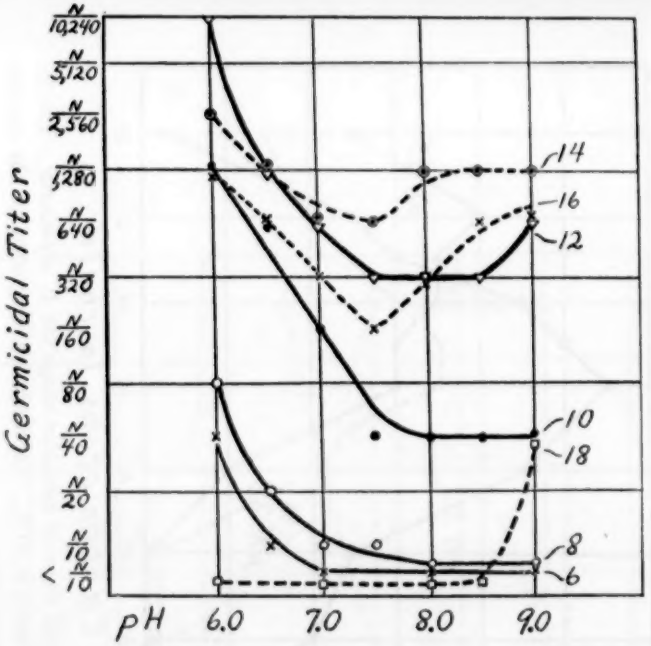

FIG. 2.

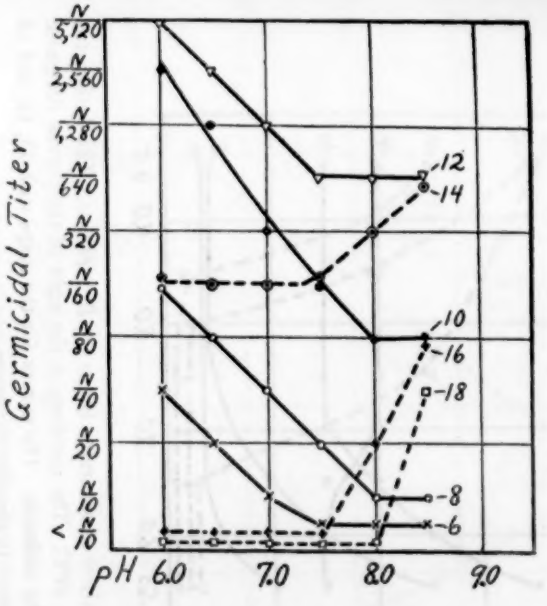

FIG. 3.

FIG. 2. Germicidal titers of saturated soaps for *Streptococcus pyogenes*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

FIG. 3. Germicidal titers of saturated soaps for *V. choleræ*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the undecylate, tridecylate, and pentadecylate are omitted; they would occupy an intermediate position between the adjacent soaps with an even number of carbon atoms.

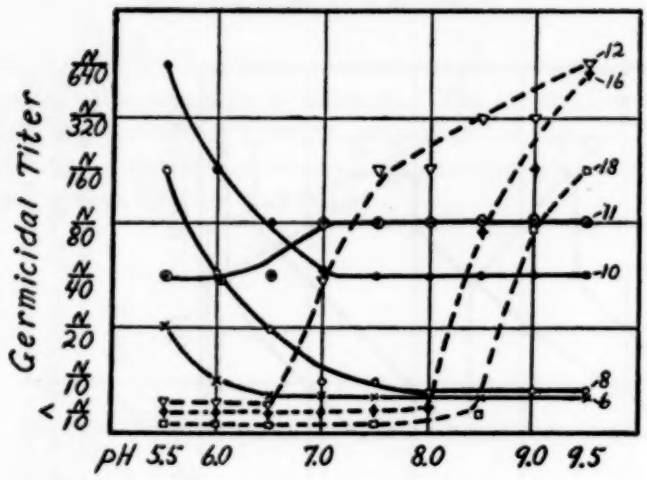

FIG. 4. Germicidal titers of saturated soaps for *B. typhosus*. The incubation period was 2 hours at 37°C. The numbers at the right of the curves designate the carbon atoms in the molecule. The curves for the soaps with 13, 14, and 15 carbon atoms were omitted to simplify the figure.

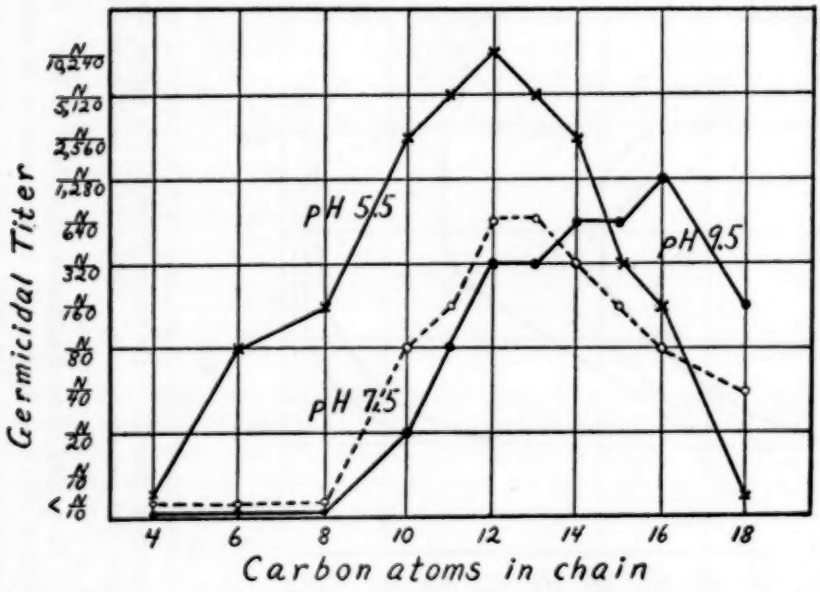

FIG. 5. The germicidal titers of saturated soaps for *B. diphtheriæ*. The incubation period was 2 hours at 37°C. The soaps are designated by the number of carbon atoms in their molecule.

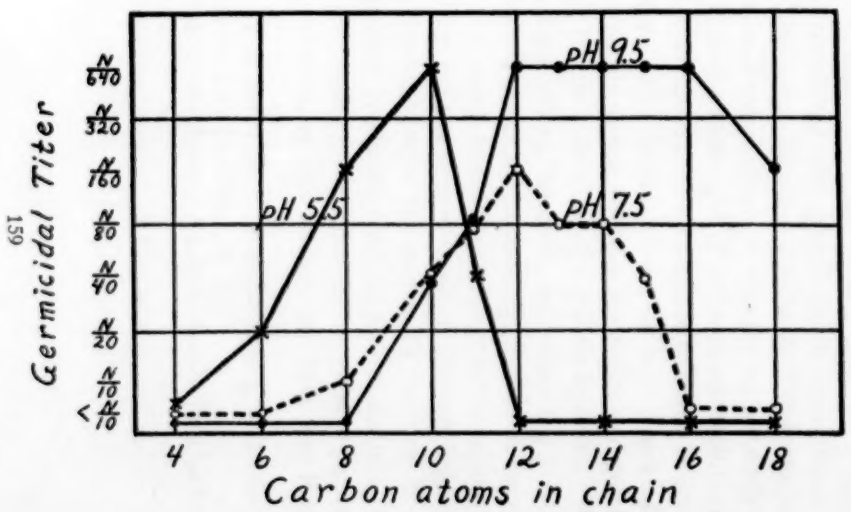

FIG. 6.

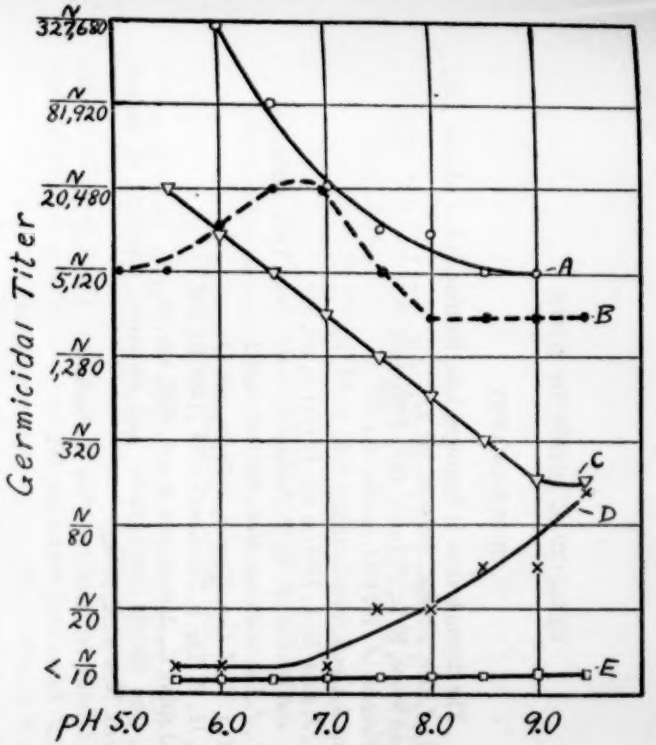

FIG. 7.

FIG. 6. The germicidal titers of saturated soaps for *B. typhosus*. The incubation period was 2 hours at 37°C. The soaps are designated by the number of carbon atoms in their molecule.

FIG. 7. The germicidal titers of two unsaturated soaps. The incubation period was 2 hours at 37°C. *A*. Potassium oleate and *Streptococcus pyogenes*. *B*. Potassium oleate and *B. diphtheriæ*. *C*. Potassium ricinoleate and *B. diphtheriæ*. *D*. Potassium oleate and *B. typhosus*. *E*. Potassium oleate and *Staphylococcus aureus*.

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THE SWELLING PRESSURE OF GELATIN AND THE MECHANISM OF SWELLING IN WATER AND NEUTRAL SALT SOLUTIONS.

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The swelling of gelatin when placed in aqueous solution may be readily separated into three types. The swelling in acid or in alkali and the effect of neutral salts on this swelling have been shown by the work of Procter and Wilson,[1] and Loeb[2] to be due to the osmotic pressure of the ions of the electrolyte, in accordance with the Donnan equilibrium. The initial swelling of dry gelatin in water—which evidently is not connected with the Donnan equilibrium—has been carefully studied by Katz,[3] who was able to show that the heat effects, volume, pressure, and vapor pressure changes were strictly analogous to those observed in the formation of concentrated solutions of many substances, and that the system as a whole behaved as an ideal concentrated solution. The large amount of heat liberated indicates strongly that a reaction occurs between the water and gelatin, resulting, presumably, in the formation of a gelatin hydrate. When sufficient water has been added, however, to reduce the gelatin concentration to less than 50 per cent, the heat effects become very small and yet the gelatin may swell, under favorable conditions, until the concentration of gelatin is 5 per cent or less. It appears improbable that this water is all combined with the gelatin in the form of a hydrate, and it seems necessary to consider this as a third distinct type. It is this type of swelling which is discussed in the present paper.

The influence of salts on this tertiary swelling was found by the

[1] *Cf.* Wilson, J. A., in Bogue, R. H., The theory and application of colloidal behavior, New York and London, 1924, i, 1.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[3] Katz, J. R., *Kolloidchem. Beihefte*, 1917–18, ix, 1.

writers[4] to be closely parallel to the effect of salts on the osmotic pressure of gelatin solutions, and it was further shown that these effects could not be accounted for on the basis of a Donnan equilibrium. It was suggested that this swelling was a purely osmotic phenomenon and that the effect of salts was due primarily to their influence on the osmotic pressure. It is evident that the problem would be greatly simplified if the pressure with which the water was drawn into the solid gelatin could be measured instead of merely the rate or extent of swelling. This "swelling pressure," although analogous to osmotic pressure of a gelatin solution, cannot be measured in the same way, since if solid gelatin is enclosed in a rigid membrane the pressure will be exerted on the walls of the membrane. It was found possible however, by placing the gelatin outside a porcelain thimble coated with collodion, to measure this swelling pressure. The measurements show that the pressure measured in this way is but little less than the osmotic pressure of the same gelatin when liquid, and they corroborate the idea that the swelling in water and neutral salt solutions is due to the osmotic pressure of the solution held in the meshes of the gel.

Experimental Method.

Gelatin.—Isoelectric gelatin was prepared as described by Loeb[2] and all measurements were made at pH 4.7.

The method of making the measurements is shown in Fig. 1. The Chamberland filter was coated with collodion by pouring collodion slowly on the surface while the thimble was rotated mechanically. The tube was partially filled with gelatin of the desired concentration and the thimble and manometer tube, previously filled with water, inserted as shown in the figure. The tube was then placed in a water bath at the desired temperature and the pressure measured after equilibrium was reached. A number of experiments were made to determine whether the thickness of the gelatin layer or the previous treatment of the system had any influence on the final reading. The results of some of these experiments are shown in Table I. The equilibrium pressure is evidently independent of the thickness of the gelatin layer, and the temperature effects are reversible. It was also found that the final pressure was independent of the initial pressure; *i.e.*, it is a true equilibrium value. The results, when the tube was completely filled with gelatin were much more regular, and this method was used in most of the experiments. The figures are the averages of 4 to 8 measurements and are reliable to about 5

[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, viii, 317.

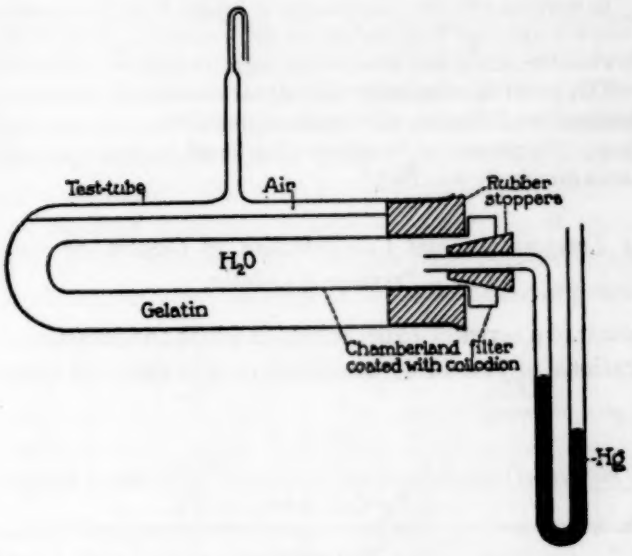

FIG. 1. Apparatus for measuring swelling pressure of gelatin.

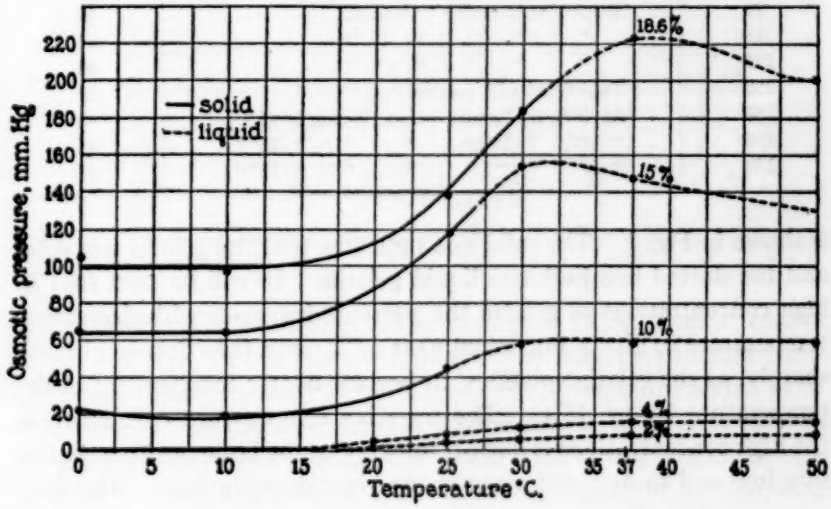

FIG. 2. Osmotic or swelling pressure of various concentrations of gelatin at different temperatures.

per cent. In working with the thin coatings of gelatin it was occasionally noticed that the water evaporated from the gelatin and condensed on the surface of the tube even when the manometer showed high negative pressure on the water in the thimble. This result is exceedingly difficult to account for except on the basis of some temperature difference, and is perhaps similar to von Schroeder's[5] anomalous findings. The pressure of the gelatin when liquid was determined in a rocking osmometer as previously described.[4]

Effect of Temperature and Concentration of Gelatin on the Swelling Pressure of Gelatin.

The result of a series of experiments in which the pressure of various concentrations of gelatin as measured over a range of temperatures

TABLE I.

Effect of Thickness of Gelatin Layer and of Previous Treatment on Swelling Pressure of 10 Per Cent Gelatin at 25°C.

Gelatin on thimble.	Other conditions.	Pressure.
cc.		*mm.Hg.*
25	Suspended in closed tube in air.	49
48	" " " " " "	54
195	" " " " " "	44
250	Tube filled with gelatin.	45
250	At 0°C. for 10 min. before placing at 25°C.	48
250	" 0° " " 18 hrs. " " " 25° "	46
250	" 37° " " " " " " 25° "	47

is shown in Fig. 2. The solid line indicates that the gelatin was solid and the dotted line indicates liquid gelatin. It will be seen that in high concentrations of gelatin the pressure increases with decreasing temperature to give a maximum near 37°C., and then decreases quite sharply, as the gelatin solidifies, to become nearly independent of the temperature below 15°C. The negative temperature coefficient in the high concentrations is due presumably to the large positive heat of solution[6] and so does not occur in the low concentrations. The drop

[5] von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 109.

[6] *Cf.* Findlay, A., Osmotic pressure, London and New York, 2nd edition, 1919, 58.

in the pressure between 30° and 20°C. occurs also in the low concentrations where the gelatin does not solidify, and in these cases approaches zero. In these dilute solutions a precipitate forms and hence no osmotic pressure would be expected. If gelatin is considered as a single chemical substance, these results are evidently exceedingly difficult to understand. The drop in the pressure below 35°C. would result if an insoluble substance is separating out, but if the solution contained only this substance the pressure must become independent of the initial concentration as soon as the solid phase appears. This

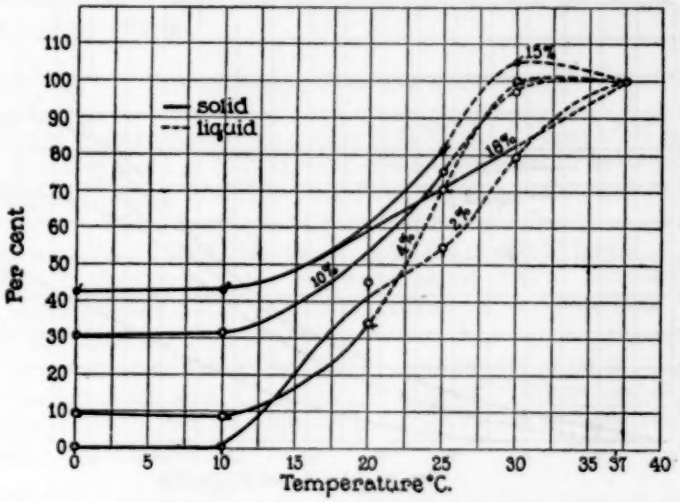

FIG. 3. Osmotic or swelling pressure of gelatin of different concentrations as per cent of value at 37°C.

is not the case. The experiment is analogous to Sörensen's[7] results on the solubility of the globulins, in that the osmotic pressure (concentration) increases with the total amount of substance added even in the presence of the solid phase. This is the expected result if it is considered that gelatin is a mixture of (at least) two substances, one of which is easily soluble over the range of temperature studied, and the other of which is readily soluble above 35°C., but much less soluble below 15°C. The drop in the pressure between 35° and 15°C. is due

[7] Sörensen, S. P. L., Proteins, The Fleischmann Laboratories, New York, 1925.

then to the separation of the insoluble fraction. If the concentration is sufficiently high a gel results; if not, particles are formed containing the soluble material and there is no osmotic pressure of the solution as a whole. The osmotic pressure as per cent of the value at 37°C. is plotted in Fig. 3 and as a function of the concentration of gelatin in Fig. 4. The percentage drop in the osmotic pressure is nearly constant in this range except for the very low concentrations. It will be noticed that the osmotic pressure increases more rapidly than the concentration, a result which can be predicted from the viscosity

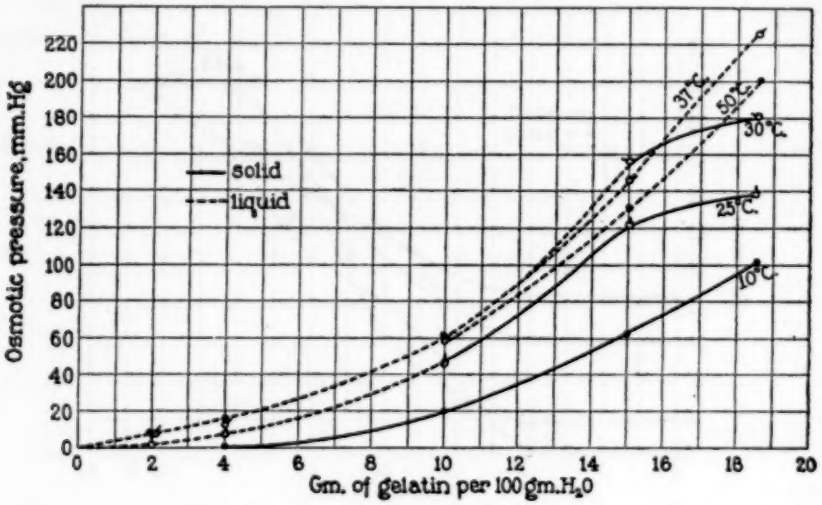

FIG. 4. Concentration and swelling or osmotic pressure of gelatin at different temperatures.

curves (Kunitz[8]) and which is due to the fact that a considerable quantity of the water is contained in the particles. (This effect will be discussed quantitatively in a subsequent paper.) The pressure also increases with the total concentration even when the gelatin is solid. This shows, as stated above, that the system cannot be considered as a saturated solution of one substance.

It should be possible, according to these results, to separate gelatin into two fractions: a soluble one, having high osmotic pressure, low

[8] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 715.

viscosity, and no tendency to gel formation, and an insoluble one, having a limited solubility at low temperatures, a low osmotic pressure, and undergoing little or no swelling. It might also be expected, since the soluble fraction must be held in the meshes of the insoluble one, that isolation of the former would be much more easily accomplished than the separation of the insoluble from its accompanying soluble component. It was found possible after a number of attempts to separate gelatin into two fractions having nearly the properties described above. This was done by combining the temperature effect on the solubility with alcohol precipitation. The fractionation was carried out as follows:

Preparation of Insoluble Fraction.

10 liters of 5 per cent isoelectric gelatin at 35°C. and 7.5 liters of 95 per cent alcohol added; 18 hours at 20°C., filtered with folded paper. Filtrate = No. 1.

Precipitate: Made up to 8 liters with water, heated to 35°C., and 2 liters of alcohol added. Cooled to 20°C. and filtered. Filtrate rejected.

Precipitate: Made up to 8 liters, etc. as above, and repeated 4 times.

Precipitate: Made up to 2 liters with water, heated to 35°C., and 1.6 liters of alcohol added. Cooled to 30°C. Jelly-like residue settled. Supernatant liquid decanted and rejected.

Residue: Made up to 2 liters, etc. as above, and repeated 5 times.

Final residue: Made up to 2 liters with water at 35°C., 800 cc. alcohol added, and cooled to 10°C. Precipitate filtered and dried with alcohol and ether. 42 gm. marked "insoluble fraction."

Preparation of Soluble Fraction.

Filtrate No. 1: 24 hours at 0°C. and filtered at 0°C. Filtrate rejected.

Precipitate: Made up to 400 cc. with water, heated to 30°C., 400 cc. of alcohol added, and cooled to 20°C. Filtered. Precipitate rejected.

Filtrate: 18 hours at 0°C. Filtered at 0°C. Filtrate rejected.

Precipitate: Made up to 200 cc., heated to 30°C., and 200 cc. of alcohol added. No precipitate on cooling to 20°C. Cooled to 0°C. 18 hours. Filtered at 0°C. Precipitate dried with alcohol and ether. 25 gm. marked "soluble fraction."

No evidence is at hand to show that either of these fractions is a chemical individual and on the contrary there is reason to suppose that the insoluble fraction still contains a considerable quantity of the soluble since it still swells, and further purification caused a still further

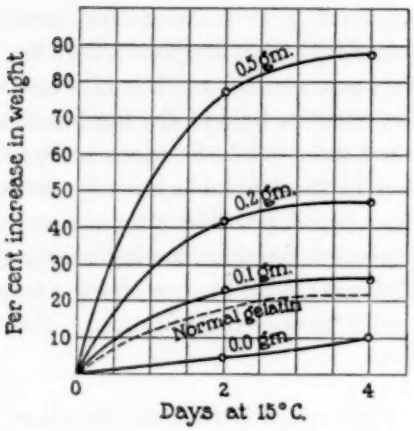

FIG. 5. Effect of increasing amounts of soluble fraction on swelling of insoluble fraction. 5 cc. H_2O, 0.4 gm. insoluble + noted amounts of soluble fraction at 15°C.

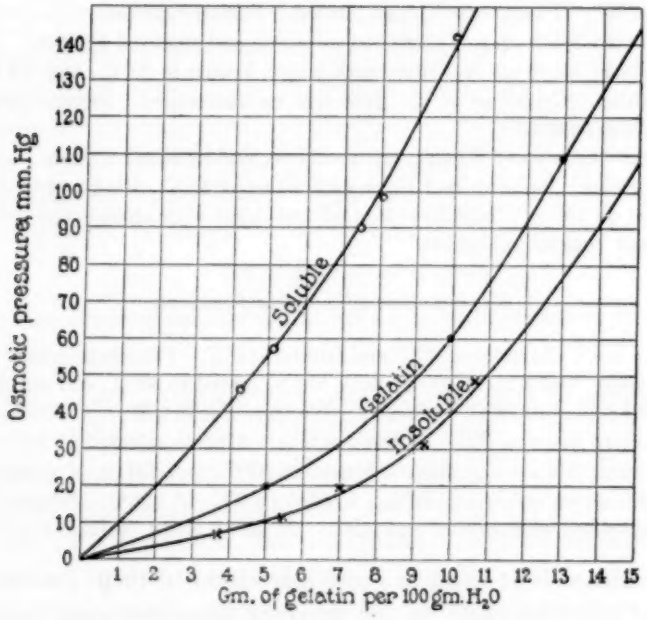

FIG. 6. Osmotic pressure of various concentrations of gelatin and of the soluble and insoluble fractions.

decrease in the osmotic pressure. Owing to the difficulty of obtaining sufficient material with which to work, however, the process was stopped at this stage.

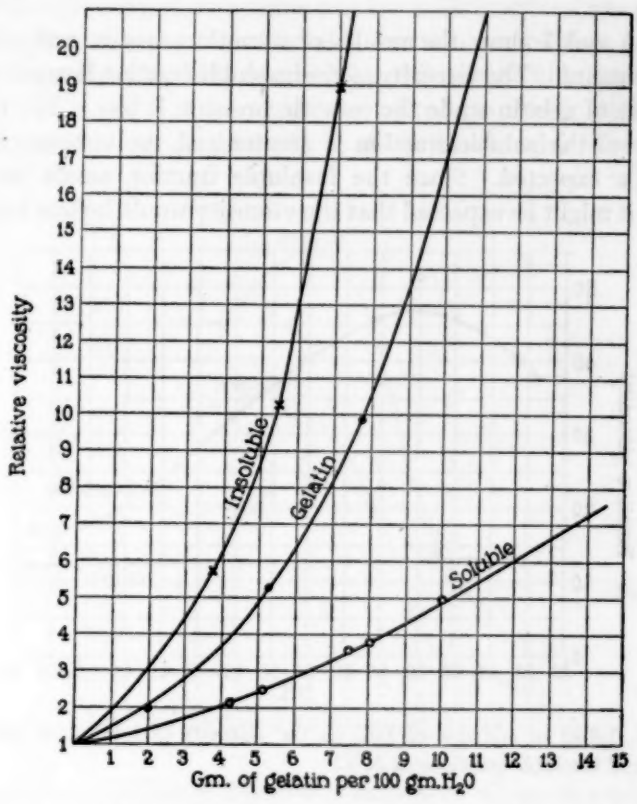

FIG. 7. Viscosity of various concentrations of gelatin and of the soluble and insoluble fractions.

Comparison of the Properties of the Two Fractions and of Gelatin.

In accordance with the mechanism of swelling outlined above it would be expected that the insoluble fraction should swell very little while the soluble fraction should dissolve. Mixtures of the two should swell the more the greater the percentage of soluble material. Fig. 5 shows that this is actually the case. The swelling of the in-

soluble fraction itself is probably due to the fact that it still contains an appreciable quantity of the soluble material, although it could of course be assumed that a different mechanism was responsible for this swelling.

Figs. 6 and 7 show the results of osmotic pressure and viscosity measurements. The viscosity of the insoluble fraction is much greater than that of gelatin while the osmotic pressure is less. The osmotic pressure of the soluble fraction is greater and the viscosity less, as would be expected. Since the insoluble fraction swells less than gelatin, it might be expected that the viscosity would be less instead of

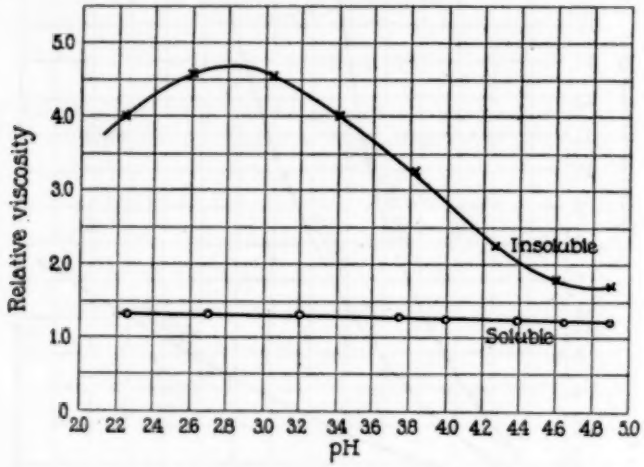

FIG. 8. Effect of addition of HCl on the viscosity of 1 per cent solutions of soluble and insoluble fractions at 37°C.

greater. It must be remembered, however, that although the individual particles swell less, the number of particles per gm. is greater in the insoluble fraction than in gelatin, since the latter also contains the soluble material. Since the viscosity, according to Einstein, depends on the total volume occupied by the particles, the higher viscosity of the insoluble fraction is due to the fact that the increase in the number of particles capable of swelling more than makes up for the difference in the swelling of the individual particles. As was stated above, the form of the osmotic pressure curves can be quantitatively accounted for by the absorption of water in the particles. Qualitatively it can

be predicted that the substance having the highest viscosity should have the greatest curvature in the osmotic pressure-concentration curves and, as the figures show, this is the case. The marked difference in the viscosity of the soluble and insoluble fractions was ascribed above to the fact that the insoluble fraction in solution consisted largely of particles capable of swelling, while the soluble fraction did not. If this is the case, it would be expected that the insoluble fraction should show a marked pH-viscosity effect, while the soluble

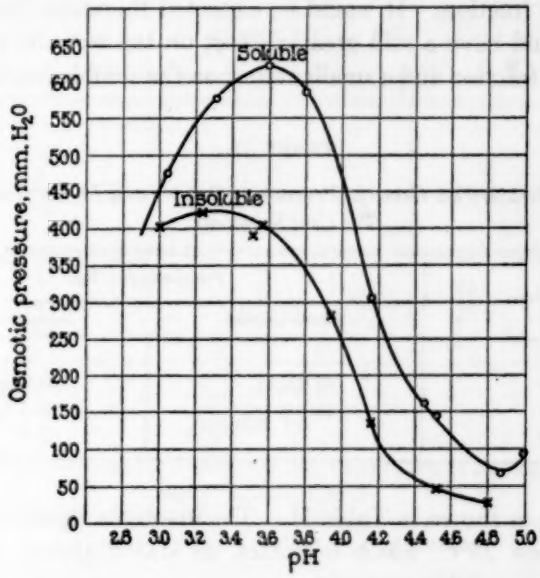

FIG. 9. Effect of addition of HCl on osmotic pressure of 1 per cent solutions of soluble and insoluble fractions at 37°C.

fraction should show little or none, since, according to Loeb,[2] the increase in viscosity on addition of acid is due to the swelling of such particles by the Donnan equilibrium set up in them. Fig. 8 shows that this is the experimental result. On the other hand, it would be expected that the effect of acid on the osmotic pressure would be about the same, since in this case the collodion sac is the particle and is evidently present in both cases. This expectation is also fulfilled as is shown in Fig. 9.

The titration curves of the two fractions, and the speed with which they are digested by pepsin, do not differ significantly from those of ordinary gelatin.

The Effect of Temperature and Salts on the Osmotic Pressure.

It was suggested above that the increase of pressure occurring when gelatin solutions or gels are raised from 25° to 37°C. was due to the solution of the substance forming the network and corresponding to the insoluble fraction. It would be expected therefore that the temperature would have a still greater effect on the osmotic pressure of the insoluble fraction and a smaller effect on the soluble fraction. This

TABLE II.

Effect of Temperature on Osmotic Pressure of Soluble and Insoluble Fractions. 5 Per Cent Solutions.

Temperature.	Pressure in mm. Hg.	
	Soluble fraction.	Insoluble fraction.
°C.		
37	50 liquid.	10 liquid.
25	48 "	6.7 solid.
20		5.3

is the result, as shown in Table II. The insoluble fraction still exerts pressure below 25°C. which indicates, as stated above, that it still contains some soluble material.

As is well known, strong solutions of some neutral salts liquefy gelatin and, as was shown in a preceding paper,[4] also increase the osmotic pressure. The action of these salts on gelatin at 15°C., and the effect of raising the temperature to 37°C. are therefore similar except that the increase of osmotic pressure due to the salt is greater than that due to increasing the temperature. A solution of gelatin at 37°C. contains particles capable of swelling, as is shown by the pH-viscosity effect. The osmotic pressure of such a solution could be increased in two ways:

First, the salt might increase the swelling of the particles. Since

in this process water is removed, while the number of particles remains the same, the result is an increase in the mol fraction, and a resulting increase in the osmotic pressure. Second, the salt might cause these particles to break up into smaller ones, thereby increasing the number of particles and hence the osmotic pressure. On the basis of the first assumption it would be expected that the salt should also increase the viscosity and further that the effect of temperature, in the presence of salt, would not differ very much from that in the absence of salt. On

TABLE III.

Effect of Salts on Viscosity and Osmotic Pressure of 10 Per Cent Gelatin.

Concentration of salt.	0	2 M NaSCN
Relative viscosity, $\frac{\eta}{\eta_{salt}}$, at 37°C.	19.3	18.10
Osmotic pressure, *mm. Hg*, at 37°C.	60	122
" " " " " 25°C.	44	113
" " " " " 15°C.	20	108
" " " " " 3°C.	20	102

TABLE IV.

Effect of NaSCN on the Osmotic Pressure of 5 Per Cent Soluble and Insoluble Fractions of Gelatin at 37°C.

Fraction.	Soluble.		Insoluble.	
Concentration of NaSCN inside and outside	0	2M	0	2M
Osmotic pressure, *mm. Hg*	50	61	8	35

the second assumption there would be expected no change or a decrease in the viscosity and the temperature effect on the osmotic pressure in the presence of salt should be much less than when no salt is present. The experiments in Table III show that the latter results are obtained. 2 M NaSCN has very little effect on the viscosity of 10 per cent gelatin and the osmotic pressure decreases only slightly in the range from 37° to 3°C. The optical properties of the solution also bear out this as-

sumption, since a solution of gelatin at 37°C. shows a very marked Tyndall cone, whereas the same solution in 2 M NaSCN shows only a faint cone.

It has already been shown that the effect of temperature is largely on the insoluble component and it remains to be seen whether the effect of salt is also on this component or on the soluble fraction. Table IV gives the effect of NaSCN on the osmotic pressure of a 5 per cent solution of the soluble and insoluble fractions at 37°C. It is evident that there is a very large increase in the osmotic pressure of the insoluble fraction and only a small increase in that of the soluble one. The salt therefore affects the same constituent as does raising the temperature.

DISCUSSION.

The preceding experiments appear to the writers to furnish a basis for a simple and satisfactory picture of the mechanism of the swelling of isoelectric gelatin in water and in neutral salt solutions. The block of gelatin is a network consisting of threads of a substance insoluble in cold water and holding in its meshes a solution of a substance soluble in water. (For the sake of simplicity only two substances are considered, although in reality there are probably a series of substances whose properties grade from very easily soluble to insoluble.) The process of manufacture of gelatin would be expected to lead to the formation of just such a series of compounds, since it is formed by the hydrolysis of an insoluble substance, collagen. Such a reaction would be expected to produce a series of split products ranging in complexity from the amino-acids up to unchanged collagen. In the further process of purification and washing, all substances which can diffuse through the network of insoluble material are removed while those that cannot are held back. When the block is immersed in water the internal solution exerts osmotic pressure and water is taken in until the osmotic pressure is equalized by the elastic force of the network. A slow subsequent swelling will occur, however, owing to the fatigue of this elastic force. A quantitative expression for the kinetics of swelling has been derived on this basis and will be discussed in a subsequent paper.

Raising the temperature causes an increase in the concentration of

the soluble material and hence in the osmotic pressure of the solution and at the same time causes a decrease in the amount of insoluble material forming the fibers. The swelling, therefore, increases very rapidly with the temperature until above 35°C. the network dissolves and the block disintegrates. The insoluble material, however, still exists in the solution in the form of particles capable of swelling, as shown by the pH-viscosity effect, and containing a large amount of water, as shown by the form of the osmotic pressure-concentration curves and the viscosity curves. The addition of neutral salts to the solution acts in the same way as increasing the temperature, except that the material forming the network is broken up into smaller particles.[9] In very high salt concentrations the reverse effect occurs and the gelatin eventually precipitates. The various peculiar hysteresis effects which have been noted in connection with the swelling of gelatin are referable to the effect of the conditions on the elasticity of the network, and are similar to those observed in any elastic body. Cooling the solution results in the reverse process. The particles or molecules of the insoluble material precipitate in the form of a network—if the concentration is high enough—enclosing the solution of the soluble material. If the concentration is low, this network separates in the form of small clots which still contain nearly all of the soluble fraction. It seems quite possible that the formation of this peculiar structure rather than of an ordinary precipitate is due to the presence of the soluble material which acts as a protective colloid, and that a pure preparation of the insoluble material would form a normal precipitate. A very similar condition occurs in the solution and repurification of casein; addition of small amounts of acid or alkali to isoelectric casein results in a normal saturated solution, as Cohn[10] has shown. Precipitation of such a solution by back titration, however, gives a milky colloidal solution which may form a gel, so long as any of the casein remains in solution. When the isoelectric point is reached, where all the casein is insoluble, a normal precipitate is again formed.

[9] This is perhaps analogous to the prevention of agglutination in strong salt solutions. In the case of bacteria it was found that a marked decrease in the "cohesive force" of the particles was noted in these high salt concentrations. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[10] Cohn, E. J., *J. Gen. Physiol.*, 1921–22, iv, 697.

This mechanism also accounts for the fact that gelatin and similar substances swell only in those solvents in which they are soluble, and it predicts that the greater the solubility the greater the swelling. The swelling which occurs during the early stages of the action of enzymes on gelatin also would be predicted since the hydrolysis increases the concentration of soluble material and hence the osmotic pressure. The swelling caused by enzymes is similar to the effect of salts or higher temperatures except that it is not reversible.

The alternative hypothesis that the effect of temperature or salts is to increase the hydration of the particles and hence the osmotic pressure seems less probable, since it requires that the hydration increase with increasing temperature. Since the hydration of gelatin liberates heat this would be contrary to Le Chatelier's principle. It also fails to predict the increased swelling obtained on adding the soluble fraction to the insoluble, since the osmotic pressure and viscosity curves show that the soluble fraction is less hydrated than the insoluble.

It is evident that the structure of gelatin outlined above is quite similar to that proposed by Hardy[11] and now widely accepted, except that at least two substances are postulated. The whole mechanism agrees in detail with the theory clearly presented by Duclaux[12] in connection with the swelling of rubber.

SUMMARY.

1. A method is described for measuring the swelling pressure of solid gelatin.

2. It was found that this pressure increases rapidly between 15° and 37°C., and that the percentage change is nearly independent of the concentration of gelatin.

3. It is suggested that this pressure is due to the osmotic pressure of a soluble constituent of the gelatin held in the network of insoluble fibers, and that gelatin probably consists of a mixture of at least two substances or groups of substances, one of which is soluble in cold water, does not form a gel, and has a low viscosity and a high osmotic

[11] Hardy, W. B., *Z. physik. Chem.*, 1900, xxxiii, 327.

[12] Duclaux, J., *Bull. soc. chim.*, 1923, xxxiii–xxxiv, 36.

pressure. The second is insoluble in cold water, forms a gel in very low concentration, and swells much less than ordinary gelatin.

4. Two fractions, having approximately the above properties, were isolated from gelatin by alcohol precipitation at different temperatures.

5. Increasing the temperature and adding neutral salts greatly increase the pressure of the insoluble fraction and have little effect on that of the soluble fraction.

6. Adding increasing amounts of the soluble fraction to the insoluble one results in greater and greater swelling.

7. These results are considered as evidence for the idea that the swelling of gelatin in water or salt solutions is an osmotic phenomenon, and that gelatin consists of a network of an insoluble substance enclosing a solution of a soluble constituent.

THE EFFECT OF pH ON THE PERMEABILITY OF COLLODION MEMBRANES COATED WITH PROTEIN.

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(Accepted for publication, July 28, 1926.)

In a study of some of the factors affecting the formation of protein films on collodion membranes,[1] it was found that the amounts of protein adhering to the membranes varied with the pH in a way similar, in the case of gelatin, to the variation of the fluidity of the protein solutions with the pH. The connection between the viscosity of gelatin solutions and the swelling of gelatin particles had been clearly brought out by Loeb.[2] Dr. Northrop suggested to the writer that it would be of interest to determine whether gelatin and other proteins, when deposited as films on collodion membranes, would exhibit a rise and fall of swelling in acid or alkali, as do granules of gelatin. This has been found to be the case.

EXPERIMENTAL.

The membranes were prepared on mercury by the method already described,[1] about 2 hours being allowed for the evaporation of the solvents through the cardboard case. These membranes were of the most permeable of the types studied in the previous experiments. They were cut into disks 3.81 cm. in diameter, and were coated with protein by being soaked overnight, at 30°C., in the protein solutions. In each case 3 disks were kept overnight at 30°C. in 25 cc. of the solution contained in a wide-mouthed 50 cc. Erlenmeyer flask, and in these experiments the flasks were not agitated. The disks from each flask were washed 3 times with about 300 cc. of water at 30°C. The

[1] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–26, viii, 61.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924.

weights of the disks, with and without protein, were determined after drying overnight at 100°C.

The swelling of the protein on the membranes was studied by measuring the rate of flow, under known pressure, of water and other solutions through the protein-coated membranes in the apparatus previously used,[1] which is similar to that of Bartell and Carpenter.[3] The membrane was bathed on both sides by the solution under investigation, which was forced through the membrane by a constant pressure of mercury. The area of membrane exposed to the liquid was circular, its diameter being 2.2 cm. The rate of flow was measured with the aid of a stop-watch and a mm. scale, by observing the movement of a meniscus in a horizontal tube 0.0760 cm. in diameter. The data were reduced to c.g.s. units and are given in terms of the permeability, Q, which may be defined as the number of cc. of liquid flowing in 1 second through 1 sq. cm. of membrane under a pressure of 1 dyne per sq. cm. The temperature varied from 19 to 24°C. in different experiments.

It was found that solutions of HCl or NaOH up to 0.1 M flowed through membranes which had not been coated with protein at the same rate as water. Hence the differences observed with the protein-coated membranes are to be ascribed to an effect of the electrolyte on the protein rather than on the collodion.

Each experiment was carried out with a single disk of membrane, the determinations being made in the order of increasing concentrations of acid or alkali. Since the different membranes were not all alike, the data of the different experiments are not quantitatively comparable.

Table I shows the effect of different concentrations of HCl on the permeability of a membrane which had been soaked in a 4 per cent solution of isoelectric gelatin. At the end of the experiment 1.0 M NaCl was forced through the membrane, and this was finally displaced with water, which brought the permeability back nearly to the starting point. The experiment shows that while the acid solutions removed very little gelatin from the membrane, they changed the permeability in the direction to be expected if the effect of the acid were on the swelling of gelatin particles.

[3] Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, xxvii, 252.

Table II shows a similar experiment with NaOH. Determinations were not made in concentrations above 0.1 M because the alkali attacked the membranes so that they burst under pressure. The changes in permeability are again opposite in direction to the changes in swelling observed by Loeb.

TABLE I.

Effect of HCl on Permeability of Gelatin-Coated Membrane.

Untreated membrane, dry weight = 28.0 mg.; $Q = 26.5 \times 10^{-10}$. At end of experiment, dry weight = 49.5 mg. Control without HCl treatment, 52.0 mg.

HCl, *mols per liter.*	0	10^{-5}	3×10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}	3×10^{-1}	1
$Q\times10^{10}$..	4.22	4.21	4.20	4.18	3.68	2.38	1.62	1.33	1.49	2.63	5.28	7.95

TABLE II.

Effect of NaOH on Permeability of Gelatin-Treated Membrane.

NaOH, *mols per liter*.....	0	10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}	3×10^{-2}	10^{-1}
$Q\times10^{10}$.....	2.98	2.79	2.03	1.42	1.12	1.06	1.08	1.27

It was found by experiments with HCl and NaOH solutions containing also 0.1 M NaCl that the permeability of similar membranes could be altered by pH even in the presence of salt. Table III shows the results obtained with a gelatin-coated membrane whose permeability was measured in solutions of the glycine-phosphate-acetate buffer described by Northrop and De Kruif.[4] These solutions contained 0.125 M total electrolyte; the pH values were obtained with the hydrogen electrode after the solutions had been used for the permeability measurements. The experiment shows that even in the presence of salt the pH exerts a considerable influence on the permeability, the latter being greatest in the vicinity of the isoelectric point of the protein.

[4] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

TABLE III.

Effect of Glycine-Phosphate-Acetate Buffer (0.125 M) on Permeability of Gelatin-Coated Membrane.

Untreated membrane, dry weight = 27.0 mg.; $Q = 25.7 \times 10^{-10}$. At end of experiment, dry weight = 49.6 mg. Control without buffer treatment, 48.0 mg.

pH	3.45	3.85	4.16	4.95	5.18	5.55	5.97	6.42	6.94	7.41	8.04
$Q \times 10^{10}$	1.63	1.71	2.27	2.47	2.27	2.14	1.86	1.86	1.78	1.74	1.69

Measurements of the permeability of a gelatin-coated membrane in several solutions of HCl and H_2SO_4 showed that the permeability was decreased less by H_2SO_4 than by HCl when the comparison was made at equivalent concentrations. The changes were of the same general nature as those in Table I, but the permeability in H_2SO_4 was always somewhat higher than in HCl. This result is qualitatively in accord with the experiments of Loeb[2] on the swelling of gelatin in acids, although the difference between the effects of the two acids appears to be less in the case of permeability than in the case of swelling. Another membrane was tested in a similar way with NaOH and $Ba(OH)_2$. Again the result was qualitatively similar to the swelling experiments, the permeability being always higher in the presence of a divalent ion of opposite charge to that of the protein.

In order to determine whether the effects observed were confined to gelatin-coated membranes, a few determinations were made of the permeability in HCl of membranes which had been soaked in solutions of egg albumin, edestin, euglobulin from ox serum, and serum albumin from horse serum. A single disk coated with each protein was used for permeability measurements in HCl of the concentrations 0.001, 0.01, and 0.1 M. The amounts of adherent protein on the membranes used were 28 mg. of egg albumin, 4 mg. of edestin, 15 mg. of globulin, and 11 mg. of serum albumin. In every case the permeability was lowest in 0.01 M HCl. The egg albumin and edestin showed only small differences, but in the case of the serum proteins the effect was larger. With serum globulin the permeability in 0.01 M HCl was about two-thirds of that in the 0.001 or 0.1 M

HCl, while with serum albumin and 0.01 M HCl the permeability was less than half of the value obtained with the other two solutions.

SUMMARY.

The permeability of gelatin-coated collodion membranes, as measured by the flow of water or of dilute solutions through the membranes, has been found to vary with the pH of the solutions. The permeability is greatest near the isoelectric point of the protein; with increasing concentration of either acid or alkali it decreases, passes through a minimum, and then increases. These variations with pH are qualitatively in accord with the assumption that they are due to swelling of the gelatin in the pores of the membrane, the effects of pH being similar to those observed by Loeb on the swelling of gelatin granules. Indications have been found of a similar variable permeability in the case of membranes coated with egg albumin, edestin, serum euglobulin, and serum albumin.

TEMPERATURE CHARACTERISTICS FOR SPEED OF MOVEMENT OF THIOBACTERIA.*

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I.

The gliding movements of certain *Cyanophyceæ*, *Thiobacteriales*, and gregarines provide relatively uncomplicated types of comparable activities suitable for quantitative observation. In spite of much discussion, little is known as to the mechanism of this sort of progression. Interpretations have been advanced by a number of writers,[1] but data required for formulation of a theory of this sort of movement have been lacking. For the present, we are not concerned so much with the mechanics of the movements of *Oscillatoria*, *Beggiatoa*, and other forms creeping in ways apparently similar, but in employing the rate of this type of movement as an index of metabolic changes.

For one kind of *Oscillatoria* it has been shown (Crozier and Federighi, 1924–25) that the rate of translatory movement, in this instance uncomplicated by rotation, obeys the Arrhenius equation for change with temperature. The rates of movement at different temperatures permit evaluation of the constant E, or μ, in the equation

$$\text{Velocity} \propto e^{-\frac{\mu}{RT}},$$

where e is the Napierian base, R the gas constant, and T the absolute temperature. The value of μ obtained in the experiments cited was 9240.

In these observations the light intensity was practically constant.

* Support from the Milton Research Fund of Harvard University is gratefully acknowledged.

[1] For recent views consult Fechner (1915); Schmid (1918, 1923); Prell (1923, *a* and *b*); Krenner (1925).

To discover the way in which the magnitude of E might be dependent upon light intensity, it was desired to know, among other things, the relations between speed of movement and temperature in a form free from effects due to photosynthetic activity. For this reason, in part, we turned to the leuco-thiobacteria. It is found that the relation of motility to temperature in *Beggiatoa* and *Thiothrix*, as in *Oscillatoria*, points clearly to the controlling influence of chemical processes. The values of the critical increments (E, or μ) in fact agree sufficiently with those repeatedly obtained for other activities of various organisms, and specifically for catalyzed respiratory oxidations (*cf.* Crozier, 1924–25). The value of E derived for speed of movement in *Beggiatoa* thus emphasizes the fact that the magnitude obtained with *Oscillatoria* (Crozier and Federighi, 1924–25) does not accord with any commonly encountered (Crozier, 1925–26, *b*) in connection with biological processes adequately studied. The investigation of the relation between E and light intensity is therefore expected to yield interesting suggestions as to the significance of the critical increment for movement in the case of *Oscillatoria*. This will be discussed in a subsequent paper. In the meantime, it may be pointed out that the movement of *Beggiatoa* appears to be governed by chemical processes similar to those revealed in a number of other vital activities; the details of the relationship between speed of translatory movement and temperature show certain features of general interest for this method of analysis.

II.

The organisms employed for the measurements were kept in shallow culture dishes containing the usual variety of forms occurring in brackish water putrefactive sulfureta (*cf.* Bavendamm, 1924; Baas-Becking, 1925). Two species, identified as corresponding to *Beggiatoa alba* and to a species of *Thiothrix* (*T. tenuis ?*), were taken for study from particular spots in one culture. Thin smears were mounted between two cover glasses, the lower one small enough to be placed within the glass ring of a van Tieghem cell. The cell was sealed with paraffin or chicle, and had in it a small volume of the culture liquid. The sealing was necessary to prevent dilution when the preparation was submerged in a thermostat; access of tap water

caused cessation of progression movements. The cell was mounted in a mechanical stage on the platform of a microscope so adjusted as to have the preparation submerged to a depth of 10 cm. in a water thermostat. The mechanical stage and the fine adjustment of the microscope were controlled by suitable attachments projecting above the water level. With good stirring no difficulty was experienced in maintaining desired temperatures. Light from a housed tungsten bulb was reflected from a mirror beneath the microscope. Variations in light intensity were apparently without effect on the movements of the sulfur bacteria, but for practically all of the measurements the light was of approximately 30 m. c. intensity. Within periods of 6 hours or longer, even up to 24 hours, no progressive changes in speed of movement were detected. Hence the sealed atmosphere in the observation cell produced no special effect.

The measurements were made of the longitudinal progression of straight filaments, and so far as could be determined in the absence of mechanical impedance. With a 5 mm. objective and 7.5 × ocular, ten divisions of the ocular micrometer used corresponded to 0.05 mm. The time required for each filament to traverse this distance was taken with a stop-watch, a number of readings being secured at each temperature. With each preparation used, precautions were taken, through time records and by reversing the sequence of temperature changes, to insure the absence of irreversible thermal effects.

III.

According to the current understanding of the mechanism of movement in *Oscillatoria* the longitudinal membrane (Hinze, 1902) of a filament is pierced by pores, through which a carbohydrate mucus is extruded (Fechner, 1915; Schmid, 1918, 1923; Prell, 1921, *a;* Krenner, 1925; Ruhland and Hoffmann, 1925). This would account for the phenomena which gave rise to the older conception of "extracellular protoplasmic streaming." Another view regards the movement as due to "modifications of surface tension," perhaps caused by osmotic processes (Coupin, 1923); though suggestive, no particularly relevant evidence supports this idea. Krenner (1925) found the speed of translatory movement of *Oscillatoria* to vary inversely with the diameter of the species, and that the osmotic pressure of the proto-

plasts is higher in the narrow forms (measured by plasmolytic shrinkage method). Krenner therefore supposes that the specific speed of motion is determined by the turgor. For *Oscillatoria* and its relatives it is known that in general the stouter forms are the more slowly moving. On this basis, one might rather expect the specific speed to be determined by some relationship of surface to bulk. But among the sulfur bacteria we find that with forms occurring side by side in the same culture, the larger species move more quickly,—for two forms, in about the ratio of 1 to 1.5, at the same temperature, when the filament diameters are in the ratio 3.22:1.

We are by no means clear as to the meaning of the optical evidence for "extracellular protoplasmic streaming" (*cf.* also Crozier and Federighi, 1924–25; and Krenner, 1925), nor as to the homology of the superficial slime-covering in *Beggiatoa mirabilis* (Hinze, 1902; Ruhland and Hoffmann, 1925), which we have also observed, with surface structures in the forms we have employed for measurements of speed of movement.

According to Schmid (1923), who studied fragmented filaments, all parts of a filament of *Oscillatoria* are motile. Prell (1921*a*, *b*,) found that the cells of a filament "cooperate," although there seems to be no conduction of stimuli from one part of a filament to another. This agrees with the observation (Crozier and Federighi, 1924–25) that the speed of movement does not vary with the length of the filament. Mr. E. S. Castle has made similar observations on *Anabæna*. It has been noticed, however, that very small groups of cells do not move (Krenner, 1925). In *Beggiatoa* very short fragments, even comprising but three to five cells, do move, but only for very short distances; the the frequency of reversal in direction is very high. It is to be noted, as bearing upon unity of action in long filaments, that there is frequently apparent a failure of the parts of a filament to cooperate. With very long filaments (2 mm.), the two terminal regions may be moving in opposite directions; or a hook bend at one end may be moved forward bodily, in such fashion as to indicate that the bent tip region is not at all contributing to the movement. Similar cases occur in which reversal of direction of movement is not synchronous over the whole filament (*cf.* also Keil, 1912). Aside from their bearing upon the mechanism of movement, these points are of practical moment for

the measurement of speeds of progression under comparable conditions.

The speed of movement declines as the culture containing the thiobacteria ages and the cells of the organisms become vacuolated. During the most active period of growth the speed of translatory movement is quite sufficiently uniform to permit significant measurements. The speed is independent of the length of the filament. Successive estimations with a single filament show satisfactory constancy, as may be illustrated by several sets of readings:

Filament.	Temperature, t	Time to travel 10 micrometer divisions.
	°C.	sec.
A	10.2	34.6
		38.6
		39.8
		39.0
		36.8
B	16.8	23 8
		22.2
		21.8
		22.2
C	19 3	14 4
		17 6
		17.2
		18.0

It could not be shown that these slight variations are related to the incidence of reversals of direction.

The latitude of variation in such series does not significantly differ from that in series obtained from a number of different filaments. This was tested sufficiently to ensure the possibility of employing averages based upon measurements with a number of filaments. It is practically impossible, however desirable, to obtain readings over a range of temperatures from single filaments. There is indication of fluctuating variation in speed of movement, not correlated with time of day, in which the period is rather long. This is in part responsible for the scatter of the plotted means (Fig. 1). The probable error of the plotted means is less than 5 per cent of the corresponding means

(usually less than 4 per cent). For purposes of the present account we have employed data from filaments in one culture, between January 14 and January 28, 1926. Throughout this period no systematic changes in speed of movement were detected. The number of observations was 431.

IV.

The results are plotted in Fig. 1. Contrary to the case of *Oscillatoria* (Crozier and Federighi, 1924–25), the motion of *Beggiatoa* ex-

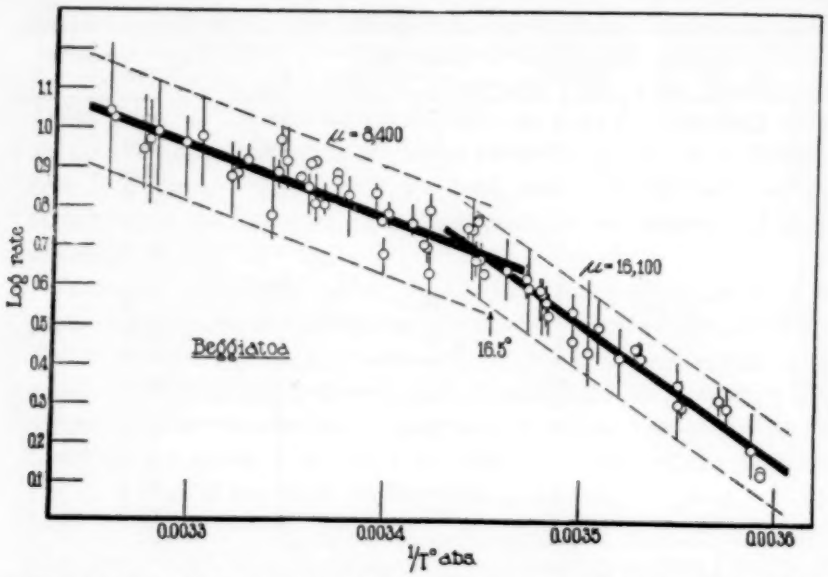

FIG. 1. Speed of gliding motion of *Beggiatoa* as function of temperature. The circles give positions of average speeds in series of readings; the vertical lines extending from them cover the latitude of variation in each series. The rate is taken as 100/ time to travel 10 micrometer divisions (0.05 mm.).

hibits a sharp change in the relation to temperature at about 16.5°. The lines providing a satisfactory fit to the two portions of the log speed–$1/T°$ *abs.* graph have slopes respectively, yielding $\mu = 8{,}400$ and $\mu = 16{,}100$ as temperature characteristics.

It may be suggested that the data are equally well fitted by a single unbroken curve. To this there is definitely opposed the fact that in such a case the slopes of the fitted straight lines could not very well be

expected to agree with those found in numerous cases where a single rectilinear relationship holds over the whole of the temperature scale. The impossibility of describing such series of observations by means of a single smooth curve is adequately shown by plotting *rates* against centigrade temperatures; the points fall upon two sharply intersecting curves. And there is also to be emphasized the fact that the temperature at which intersection of the proposed straight lines is located, as determined solely by the distributions of the relevant points, agrees so closely with one at which such irregularity is commonly or very fre-

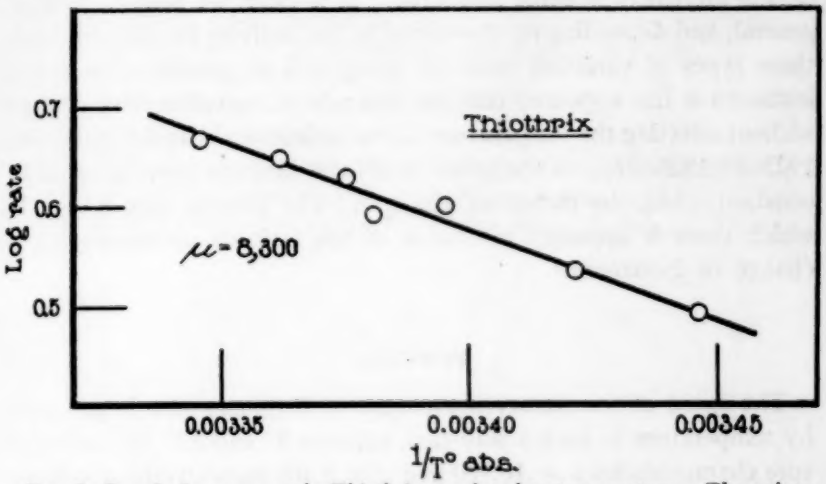

FIG. 2. Speed of movement in *Thiothrix* as related to temperature. The points are averages of 6 to 8 measurements each.

quently manifest in other vital processes (Crozier, 1925–26, *b*). Additional considerations justifying this procedure are discussed in another place (Crozier and Stier, 1926–27). Less extensive observations on the movement of *Thiothrix* provide data for the graph in Fig. 2. The temperature characteristic, $\mu = 8{,}300$, agrees well with that for the corresponding temperature range with *Beggiatoa*.[2]

[2] As with *Oscillatoria*, question also arises here as to the character and mechanism of reversal in direction of movement. According to Coupin (1923) *Oscillatoria* filaments, on Knop medium to which gelose had been added, show no regular periodicity in the reversal of movement; but it is necessary to maintain constant conditions of light and temperature before the matter can be tested. It is clear

In addition to the occurrence of a critical "break" at 16°, the temperatures 5.3° and 33° were established at points at which progressive slowing of movement with time becomes evident; at 33° or above "jerky" side to side movement is evident, with little forward motion.

The "break" at 16° is made obvious in another way. The latitude of variation at temperatures below 16° is definitely less than at higher temperatures. For some time it has been desired to find instances in which it might be possible to discover if the latitude of variation is a property of the organism or tissue as a whole, or of the process whose critical increment is being measured. It is clear, we believe, that in general, and depending on the nature of the activity considered, both these types of variation must be recognized as possible. In many instances it has appeared that the latitude of variation may change without affecting the temperature characteristic (*e.g.*, Crozier and Stier, 1925–26, 1926–27); on the other hand, the latitude may be sensibly constant when the increment changes. The present case is one in which there is apparent alteration of the latitude accompanying a change of increment.[3]

V.

SUMMARY.

The speed of translatory movement of *Beggiatoa alba* is governed by temperature in such a way that between 5° and 33° the temperature characteristics $\mu = 16{,}100$ and $\mu = 8{,}400$ respectively obtain for the temperature ranges 5° to 16.5° and 16.5° to 33°. The "break" at 16°–17° is emphasized by the occurrence of a wider latitude of variation in speed above this temperature. Above 16° the progression of *Thiothrix* yields $\mu = 8{,}300$. The possible relation of these values to that previously obtained for similar movement in (photosynthetic) *Oscillatoria* is commented upon.

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that the frequency of reversal is related to the length of the filament, being greater with short filaments, and certainly it increases with elevation of temperature. Reversal is more frequent in *B. alba* than in *Thiothrix*, under the same conditions. In forms we have observed the frequency of reversal has a higher temperature coefficient than the speed of translation.

[3] This may also be the case with the locomotion of *Paramecium* (Glaser, 1925–26).

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STEREOTROPISM IN RATS AND MICE.

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I.

With diplopods (Crozier and Moore, 1922–23) and with larvæ of *Tenebrio* (Crozier, 1923–24, *a*, *b*) it has been shown that posterior unilateral contact of the creeping animal with the edge of a thick glass plate forces the head to turn in the direction of contact. Contact with two lateral surfaces of equal extent prevents stereotropic bending, and the animal proceeds in a straight path. It was also found, especially with *Tenebrio* larvæ, that stereotropic orientation due to unilateral contact, particularly at the anterior end, persists briefly after cessation of the contact; and that unequal bilateral contacts lead to orientation through an angle roughly proportional to the difference in areas of contact.

These observations can be repeated with a variety of forms, land isopods among others, and show the purely tropistic character of such orientations during creeping. The present experiments are concerned with rats and mice. In all essential details the results duplicate those obtained with arthropods. Their special interest lies in the fact that they demonstrate the occurrence of simple tropistic behavior in mammals. The effects of memory images (Loeb, 1918), and of the great enrichment of sensory fields and their central projections (Parker, 1922), enormously increase the number of possible responses which higher vertebrates may exhibit, and prediction of the course of movements is correspondingly restricted. In order to observe tropistic conduct in a mammal, it is necessary to deal with a type of response which dominates the animal's conduct so strongly as to exclude the influence of stimulations not directly connected with this particular aspect of behavior. The stereotropism of young rats and mice fulfills this condition.

The behavior of the young opossum at birth appears to give an instance of geotropism. After being licked free of blood and embryonic membranes, they climb "hand over hand" from the genital opening to the pouch (a distance of about three inches), and attach themselves to the teats (Hartman, 1920). "If the skin be tilted, the embryos can be made to travel upward and even *away* from the pouch for they are negatively geotropic." The postural reflexes of the decerebrated rabbit (Magnus, 1915–16) include responses which may be taken to have a basis in stereotropism. If placed on the ground with the body in an asymmetric position, the head moves to the normal symmetrical orientation even in the absence of otic labyrinths. This is prevented if a board is placed upon the animal lying in an asymmetrical position; when asymmetric contact-stimulation is equalized, the animal retains an abnormal position.

Accounts of the behavior of rats and mice (*e.g.* Vincent, 1911–12) contain a number of observations suggesting stereotropic guidance.[1] We desired to see if tropistic conduct could not be demonstrated more clearly. This is best accomplished by determining if, as in the case of invertebrates, stereotropic orientation during creeping might not obey the law of the composition of forces. In this event equal bilateral contacts should obliterate turning toward a source of contact. To rule out effects of vision and of the tactile rôle of vibrissæ, we have used animals lacking eyes or vibrissæ. It is neater, in so doing, to avoid experimental mutilation by employing individuals "operated upon" through the agency of a genetic factor, such as results in blindness. This we have been able to do.

II.

The animals used were young rats (*Rattus norvegicus*) and mice (*Mus musculus*) aged 9 to 20 days. The rats were albinos and dark-eyed young from a backcross of the King inbred albinos to hooded rats sired by King inbred males. The mice were all dark-eyed.

[1] Watson (1914, p. 424) seems to express a view generally held, that "the so-called 'stereotropism' which such animals exhibit is probably no more a case of stereotropism than is the action of a blind man in keeping near a wall or the edge of the side walk." On the preceding page, moreover, he considers it "strange" that when vibrissæ are removed from the *right* side, the rat keeps close to the *left* side of the path in a maze. *Cf.* also Przibram (1913, p. 99).

The eyes of young mice and rats are opened 10 to 14 days after birth. At first, only animals with unopened eyes were used; but it was soon found that young animals with opened eyes gave substantially the same results. Tests with rats were for the most part made in a dark room, under red light of low intensity, at 23–25°C. Experiments with mice were made at about the same temperature, but not in a dark room. Temperatures as low as 15° greatly reduce activity.

FIG. 1.

FIG. 2.

FIG. 1. Stereotropic orientation of young rat or mouse at the corner of a box along one side of which it has been creeping.

FIG. 2. A young rat or mouse has been creeping in contact with the side of a box (dashed outline); the removal of the box results in partial orientation toward that side.

Typical stereotropic behavior is observed in animals creeping or walking at a fairly rapid rate. During slow progression there is more opportunity for the lurching gait to induce movements which, while in the main of stereotropic origin, nevertheless interfere with diagrammatic orientation.

Contact with a vertical surface during creeping results in its being followed closely, and at the end of the surface bending is invariably seen toward the contact side. Depending upon the rate of creeping, the animal either proceeds at an angle with the path while in contact,

or if the progression has been slow, it may turn and continue to maintain contact. Fig. 1 shows the path taken after contact with the side of a box, and at its corner. If the box is suddenly removed while the animal is creeping, there is always a swerving toward the side where the box was located (Fig. 2). Stroking one side causes turning in that direction. These results are exactly similar to those gotten with arthropods.

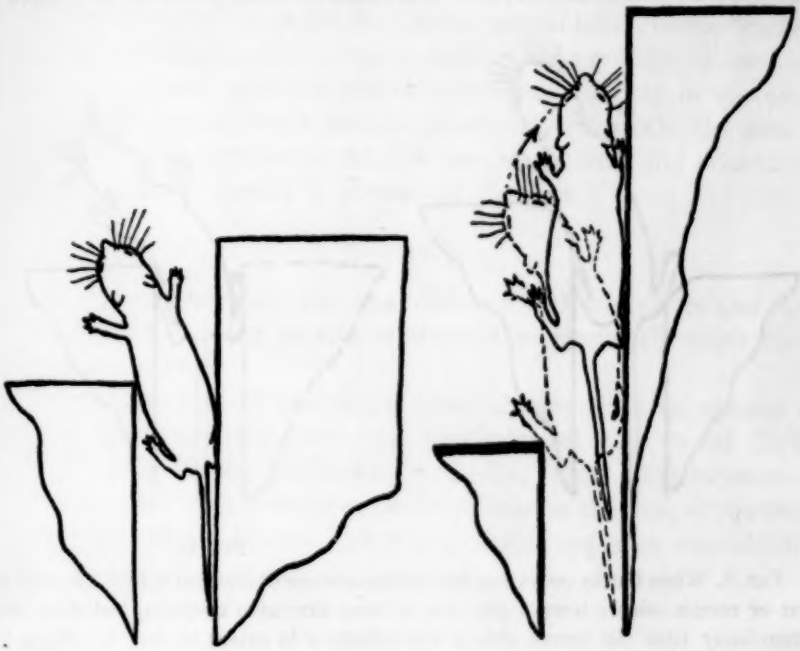
FIG. 3. A young rat or mouse creeping in a passage-way between two boxes, just wide enough to permit gentle contact on either side during the animal's swaying progression, is found to emerge from the passage-way without orientation. Equivalent bilateral stimulations prevent stereotropic turning.

When the vibrissæ have been recently cut away the creeping movements are slower and more uncertain, yet the stereotropic responses continue; several days later the uncertainty of the creeping is lost, but the animal continues to move with head held close to the floor. Removal of the tail has even less effect. Removal of both tail and vibrissæ does not materially interfere. The surface of the body and legs is thus sufficient to control stereotropism.

III.

A young rat or mouse creeping between two boxes so placed as to give equal contact on either side typically emerges from the alley-way

in a perfectly straight course (Fig. 3). The bulging of the body at the level of the hind legs, coupled with the lurching gait, sometimes causes quite unequal contacts on the two sides, and this results in modification of the path on emergence. If one box is advanced beyond the other, the animal frequently emerges at an angle toward the extended



a *b*

FIG. 4. *a*. Contact at one side with the corner of a box may lead to orientation toward that side, apparently due, in part at least, to more intense tactile excitation than is provided by a continuous flat surface (or by smoothly rounded corners; see Fig. 5).

b. When such a corner is passed, orientation persists toward a continuing contact on the opposite side.

side. This angle decreases with increase of the excess contact zone on that side. If one surface extends more than the length of the body beyond the corner of the opposed box, the mouse or rat emerges at a very acute angle and then orients so as to round the corner against the surface of the more extended box (Fig. 4). This is not exactly the

result obtained with arthropods and other forms, which emerge at an angle toward the side of more extensive contact. But further tests show that this at-first-sight anomalous result is due to the relatively excessive tactile stimulation provided by the sharp corners of the wooden boxes used in such experiments. The same outcome is generally observed if thick blocks of paraffin are used instead, provided they have

FIG. 5.

FIG. 6.

FIG. 5. When blocks providing lateral contacts are of unequal extent, the young rat or mouse orients toward the side of more extensive contacts, but does not completely turn the corner unless the difference in extent of the two blocks is more than half the length of the animal. This, the expected result from a tropistic standpoint, is obtained when the corners of the contact blocks are smoothly rounded.

FIG. 6. An individual emerging from equal bilateral contacts with two boxes (*cf.* Fig. 3) proceeds in a straight path, without orientation; but if one of the boxes be removed (dashed outline), it promptly orients toward the remaining one.

sharp corners. But if the corners be smoothly rounded, the result of such tests is entirely consonant with the interpretation that stereotropic orientation during creeping varies in amplitude according to the difference in the excitations on the two sides (Fig. 5). If one of two opposed boxes providing bilateral contact at emergence be suddenly removed, the animal orients toward the remaining surface (Fig. 6).

These experiments were repeated many times, with particular effort to obviate any persistent tendency of single individuals to right- or left-hand turning.

During creeping in contact with a single vertical surface, the young rat or mouse, especially if moving very slowly, occasionally reverses direction. Observation shows that this occurs when the opposite side makes contact with the floor, as the animal falls into the "corner" between the floor and box; this is similar to the rotation of the body on its long axis observed with invertebrates creeping in the angle between a vertical and a horizontal surface. As a rule, the area of contact is then greater on the side toward the box, and orientation in this direction results in reversal of the path.

IV.

These responses have also been obtained with adult mice and rats, but visual and other sources of stimulation frequently make them much less precise.

Mice blind through hereditary defect characterized by absence of visual cells in the retina were very kindly loaned to us by Dr. Clyde Keeler of the Bussey Institution (*cf.* Keeler, 1926). The locomotion of these adult mice is much more direct than in the case of the very young individuals, and the typical stereotropic responses were obtainable with great certainty and clearness.

V.

The reactions we have described as typical are of course not exhibited with diagrammatic clearness at every trial. The more significant sorts of deviation, however, are themselves stereotropic in origin.

The stereotropism of rats and mice as observed in these experiments was always positive. Movement away from a contact surface is occasionally seen with the younger animals, but it is easily shown that this is an accidental consequence of the method of creeping. The leg muscles are not yet well developed, and the legs are disproportionately long; the body is kept fairly close to the ground and the legs are advanced in a way which cause the rather unsteady creeping act to be a

succession of pronounced lurches. An occasional lunge removes the animal from a vertical contact surface, and if all contact has been lost it may creep away from it; usually, however, the residual effect of the contact surface is sufficient to cause reorientation toward it. In case complete separation from the vertical surface has not been followed by reorientation and return, test by contact with a new surface always shows that the animal is still positively stereotropic.

The swaying mode of progression may cause a young rat emerging from bilateral contacts to move toward one side. Thus in one series of trials, with five rats aged 20 days, each animal passed eight times between two vertical contact surfaces of equal extent; in another series eleven rats aged 12 to 14 days each passed five times through equal vertical contact zones; in thirty-one of the first forty tests, and in forty-four of the second lot of fifty-five tests, emergence was in a straight line; in the cases of deviation toward one side, it was seen that the divergence was due to a lunging in that direction rather than to an act of orientation.

When the animal moves at a fairly rapid rate the unilateral effects of lurching movements are more or less equalized. With older individuals the stronger legs make for a straighter course and the influence of lurching motion almost completely disappears. Thus animals about 25 days old show extremely regular reactions, as do the adult blind mice.

The chief sources of apparent irregularity in the stereotropic responses is found to lie in chance contacts of tail and especially of vibrissæ with the boxes employed to give contact surfaces. These variations are reduced by removal of vibrissæ and tail, but the slowness and uncertainty of progression subsequent to these operations introduce other complications and prevent precise measurement of the relation of unequal contact on the two sides to the angle of orientation.

VI.

SUMMARY.

Typical stereotropic orientation toward a lateral surface of contact is obtained in young rats and mice, and with adult mice congenitally blind. Removal of vibrissæ or tail or both does not essentially affect this response.

Equal contact on both sides of the body prevents orientation toward either source of contact. Unequal contact areas on the two sides leads to orientation toward the more extensive contact.

This behavior very exactly parallels the stereotropic conduct of arthropods, and thus provides a fairly complete instance of a tropism in mammals.

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